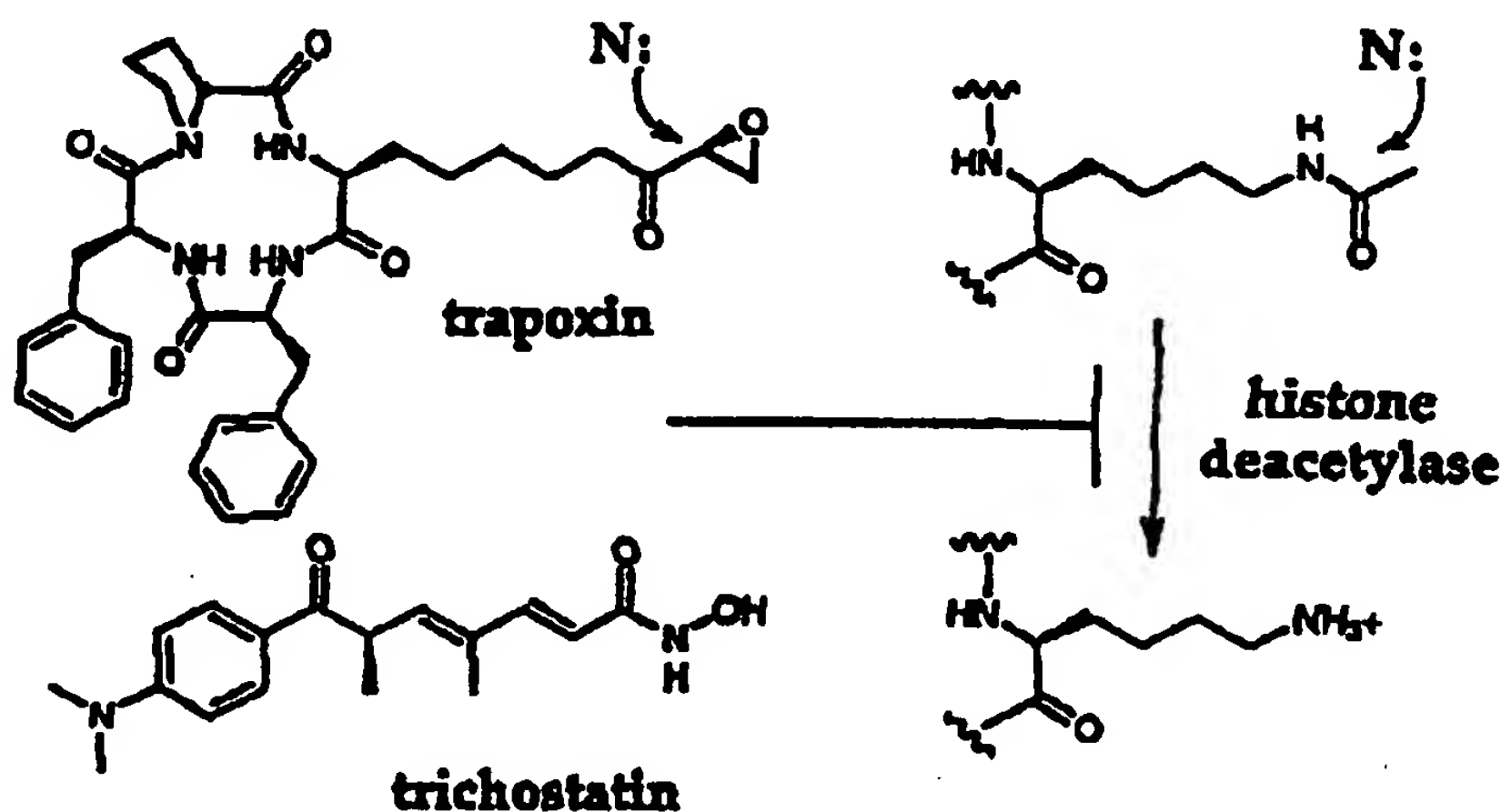




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<p>(21) International Application Number: PCT/US97/05275 (22) International Filing Date: 26 March 1997 (26.03.97) (30) Priority Data: 08/624,735 26 March 1996 (26.03.96) US (71) Applicant (for all designated States except US): PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 02138 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): SCHREIBER, Stuart, L. [US/US]; 434 Marlborough Street, Boston, MA 02115 (US). TAUNTON, Jack [US/US]; 96 Willow Avenue #1, Somerville, MA 02144 (US). HASSIG, Christian, A. [US/US]; 68R Dane Street, Somerville, MA 02143 (US). JAMISON, Timothy, F. [US/US]; 64 Frost Street #1, Cambridge, MA 02140 (US). (74) Agents: ARNOLD, Beth, E. et al.; Foley, Hoag &amp; Eliot L.L.P., One Post Office Square, Boston, MA 02109 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  Published Without international search report and to be republished upon receipt of that report.</p>

(54) Title: HISTONE DEACETYLASES, AND USES RELATED THERETO



## (57) Abstract

The present invention concerns proteins encoded by a family of genes, termed here HDx-related genes, which are involved in the control of chromatin structure and, thus in transcription and translation. The present invention makes available compositions and methods that can be utilized, for example, to control cell proliferation and differentiation *in vitro* and *in vivo*.

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## Histone Deacetylases, and Uses Related Thereto

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5 Institute of Health. The United States Government has certain rights in the invention.

### **Background of the Invention**

The organization of regulatory DNA elements into precise chromatin structures is important for both DNA replication and transcription *in vivo* (Lee et al. (1993) *Cell* 10 72:73-84; Felsenfeld (1992) *Nature*. 355:219). In eukaryotic cells, nuclear DNA exists as a hierarchy of chromatin structures, resulting in the compaction of nuclear DNA about 10,000 fold (Davie and Hendzel (1994) *J. Cell. Biochem.* 55:98). The repeating structural unit in the extended 10 nm fibre form of chromatin is the nucleosome (van Holde (1988) *Chromatin*. New York: Springer-Verlag). The nucleosome consists of 146  
15 bp of DNA wrapped around a protein core of the histones H2A, H2B, H3, and H4, known as the core histones. These histones are arranged as an (H3-H4)<sub>2</sub> tetramer and two H2A-H2B dimers positioned on each face of the tetramer. The DNA joining the nucleosomes is called linker DNA; it is to the linker DNA to which the H1 or linker histones bind. The 10 nm fibre is compacted further into the 30 nm fibre. Linker  
20 histones and amino-terminal regions ("tails") of the core histones maintain the higher order folding of chromatin (Garcia Ramirez et al.. (1992) *J. Biol Chem* 267:19587). This chromatin structure must be relaxed when DNA is transcribed or translated.

Histones of the nucleosome core particle are subject to reversible acetylation at the ε-amino group of lysines present in their amino terminus (Csordas et al. (1990) 25 *Biochem J* 265:23-38). Transcriptionally silent regions of the genome are enriched in underacetylated histone H4 (Turner (1993) *Cell* 75:5-8), and histone hyperacetylation facilitates the ability of transcription factor TFIID to bind to chromatin templates (Lee et al. (1993) *Cell* 72:73-84). Recent genetic, biochemical and immunological approaches have provided substantial evidence indicating that histones associated with actively  
30 transcribed genes are more highly acetylated than those from nontranscribed regions. While not wishing to be bound by any particular theory, histone acetylation may influence transcription at several stages, for example, by causing transcription factors to bind or by

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inducing structural transitions in chromatin, or by facilitating histone displacement and repositioning during polymerase elongation.

The acetylation and deacetylation are catalyzed by specific enzymes, histone acetyltransferase and deacetylase, respectively, and the net level of the acetylation is controlled by the equilibrium between these enzymes. The steady state level of acetylation and the rates at which acetate groups are turned over vary both between and within different cell types, with half-lives that vary from a few minutes to several hours. Although a histone acetyltransferase gene (HAT1) has been identified in yeast (Kelff et al. (1995) *J. Biol. Chem.* 270:24674-24677), the molecular entities responsible for histone deacetylation were heretofore unknown in the art.

The identification of the mechanism by which histones are deacetylated would be of great benefit in the control of gene transcription and the cell cycle.

### *Summary of the Invention*

The present invention relates to the discovery of a novel family of genes, and gene products, expressed in mammals, which genes are referred to hereinafter as the "histone deacetylase" genes or "*HDx*" gene family, the products of which are referred to as histone deacetylases or *HDx* proteins.

In general, the invention features isolated *HDx* polypeptides, preferably substantially pure preparations of one or more of the subject *HDx* polypeptides. The invention also provides recombinantly produced *HDx* polypeptides. In preferred embodiments the polypeptide has a biological activity including an ability to deacetylate an acetylated histone substrate, preferably a substrate analog of histone H3 and/or H4. In other embodiments the *HDx* polypeptides of the present invention bind to trapoxin or to trichostatin, such binding resulting in the inhibition a deacetylase activity of the *HDx* polypeptide. However, *HDx* polypeptides which specifically antagonize such activities, such as may be provided by dominant negative mutants, are also specifically contemplated.

The *HDx* polypeptides disclosed herein are capable of modulating proliferation, survival and/or differentiation of cells, because of their ability to alter chromatin structure by deacetylating histones such as H3 or H4. Moreover, in preferred embodiments, the subject *HDx* proteins have the ability to modulate cell growth by influencing cell cycle progression or to modulate gene transcription.



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In one embodiment, the polypeptide is identical with or homologous to an *HDx* protein. Exemplary *HDx* polypeptide include amino acid sequences represented in any one of SEQ ID Nos 5-8. Related members of the *HDx* family are also contemplated, for instance, an *HDx* polypeptide preferably has an amino acid sequence at least 85% homologous to a polypeptide represented by one or more of the polypeptides designated SEQ ID Nos: 5-8, though polypeptides with higher sequence homologies of, for example, 88, 90% and 95% or are also contemplated. In one embodiment, the *HDx* polypeptide is encoded by a nucleic acid which hybridizes under stringent conditions with a nucleic acid sequence represented in one or more of SEQ ID Nos. 1-4. Homologs of the subject *HDx* proteins also include versions of the protein which are resistant to post-translation modification, as for example, due to mutations which alter modification sites (such as tyrosine, threonine, serine or asparagine residues), or which inactivate an enzymatic activity associated with the protein.

The *HDx* polypeptide can comprise a full length protein, such as represented in SEQ ID No. 5, or it can comprise a fragment corresponding to particular motifs/domains, or to arbitrary sizes, e.g., at least 5, 10, 25, 50, 100, 150 or 200 amino acids in length. In preferred embodiments, the polypeptide, or fragment thereof, specifically deacetylates histone H4. In other preferred embodiments, the *HDx* polypeptide includes both a  $\nu$  motif (SEQ ID No. 12) and a  $\chi$  motif (SEQ ID No. 14), preferably a  $\nu$  motif represented in the general formula SEQ ID No. 13, and a  $\chi$  motif represented in the general formula SEQ ID No. 15.

In certain preferred embodiments, the invention features a purified or recombinant *HDx* polypeptide having a molecular weight in the range of 40kd to 60kd. For instance, preferred *HDx* polypeptides, have molecular weights in the range of 50kd to about 60kd, even more preferably in the range of 53-58kd. It will be understood that certain post-translational modifications, e.g., phosphorylation, prenylation and the like, can increase the apparent molecular weight of the *HDx* protein relative to the unmodified polypeptide chain.

The subject proteins can also be provided as chimeric molecules, such as in the form of fusion proteins. For instance, the *HDx* protein can be provided as a recombinant fusion protein which includes a second polypeptide portion, e.g., a second polypeptide having an amino acid sequence unrelated (heterologous) to the *HDx* polypeptide, e.g. the second polypeptide portion is glutathione-S-transferase, e.g. the second polypeptide portion is an enzymatic activity such as alkaline phosphatase, e.g. the second polypeptide portion is an epitope tag.

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In yet another embodiment, the invention features a nucleic acid encoding a an *HDx* polypeptide, or polypeptide homologous thereto, which polypeptide has the ability to modulate, e.g., either mimic or antagonize, at least a portion of the activity of a wild-type *HDx* polypeptide. Exemplary *HDx*-encoding nucleic acid sequences are represented  
5 by SEQ ID Nos: 1-4.

In another embodiment, the nucleic acid of the present invention includes a coding sequence which hybridizes under stringent conditions with one or more of the nucleic acid sequences in SEQ ID Nos: 1-4. The coding sequence of the nucleic acid can comprise a sequence which is identical to a coding sequence represented in one of SEQ  
10 ID Nos: 1-4, or it can merely be homologous to one or more of those sequences. In preferred embodiments, the nucleic acid encodes a polypeptide which specifically modulates, by acting as either an agonist or antagonist, the enzymatic activity of an *HDx* polypeptide.

Furthermore, in certain preferred embodiments, the subject *HDx* nucleic acid will  
15 include a transcriptional regulatory sequence, e.g. at least one of a transcriptional promoter or transcriptional enhancer sequence, which regulatory sequence is operably linked to the *HDx* gene sequence. Such regulatory sequences can be used in to render the *HDx* gene sequence suitable for use as an expression vector. This invention also contemplates the cells transfected with said expression vector whether prokaryotic or  
20 eukaryotic and a method for producing *HDx* proteins by employing said expression vectors.

In yet another embodiment, the nucleic acid hybridizes under stringent conditions to a nucleic acid probe corresponding to at least 12 consecutive nucleotides of either sense or antisense sequence of one or more of SEQ ID Nos: 1-4; though preferably to at  
25 least 25 consecutive nucleotides; and more preferably to at least 40, 50 or 75 consecutive nucleotides of either sense or antisense sequence of one or more of SEQ ID Nos: 1-4.

Yet another aspect of the present invention concerns an immunogen comprising an *HDx* polypeptide in an immunogenic preparation, the immunogen being capable of eliciting an immune response specific for an *HDx* polypeptide; e.g. a humoral response,  
30 e.g. an antibody response; e.g. a cellular response. In preferred embodiments, the immunogen comprising an antigenic determinant, e.g. a unique determinant, from a protein represented by one of SEQ ID Nos. 5-8.

A still further aspect of the present invention features antibodies and antibody preparations specifically reactive with an epitope of the *HDx* immunogen.

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The invention also features transgenic non-human animals, e.g. mice, rats, rabbits, chickens, frogs or pigs, having a transgene, e.g., animals which include (and preferably express) a heterologous form of an *HDx* gene described herein, or which misexpress an endogenous *HDx* gene, e.g., an animal in which expression of one or more of the subject *HDx* proteins is disrupted. Such a transgenic animal can serve as an animal model for studying cellular and tissue disorders comprising mutated or mis-expressed *HDx* alleles or for use in drug screening.

The invention also provides a probe/primer comprising a substantially purified oligonucleotide, wherein the oligonucleotide comprises a region of nucleotide sequence which hybridizes under stringent conditions to at least 12 consecutive nucleotides of sense or antisense sequence of SEQ ID Nos: 1-4, or naturally occurring mutants thereof. Nucleic acid probes which are specific for each of the *HDx* proteins are contemplated by the present invention, e.g. probes which can discern between nucleic acid encoding a human or bovine *HD*. In preferred embodiments, the probe/primer further includes a label group attached thereto and able to be detected. The label group can be selected, e.g., from a group consisting of radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors. Probes of the invention can be used as a part of a diagnostic test kit for identifying dysfunctions associated with mis-expression of an *HDx* protein, such as for detecting in a sample of cells isolated from a patient, a level of a nucleic acid encoding a subject *HDx* protein; e.g. measuring an *HDx* mRNA level in a cell, or determining whether a genomic *HDx* gene has been mutated or deleted. These so called "probes/primers" of the invention can also be used as a part of "antisense" therapy which refers to administration or *in situ* generation of oligonucleotide probes or their derivatives which specifically hybridize (e.g. bind) under cellular conditions, with the cellular mRNA and/or genomic DNA encoding one or more of the subject *HDx* proteins so as to inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. Preferably, the oligonucleotide is at least 12 nucleotides in length, though primers of 25, 40, 50, or 75 nucleotides in length are also contemplated.

In yet another aspect, the invention provides an assay for screening test compounds for inhibitors, or alternatively, potentiators, of an interaction between an *HDx* protein and an *HDx* binding protein or nucleic acid sequence. An exemplary method includes the steps of (i) combining an *HDx* polypeptide or fragment thereof, one or more *HDx* target polypeptide (such as a histone, SIN3, RpAp48 or other protein which participates in *HDx* complexes, e.g., one or more proteins having molecular weights of 250 kDa, 180 kDa, 55 kDa, 50 kDa, 42 kDa, 33-36 kDa and 30 kDa, see also

Example 3), and a test compound, e.g., under conditions wherein, but for the test compound, the *HDx* protein and target polypeptide(s) are able to interact; and (ii) detecting the formation of a complex which includes the *HDx* protein and target polypeptide(s) either by directly quantitating the complex, the deacetylase activity of the *HDx* protein, or by measuring inductive effects of the *HDx* protein. A statistically significant change, such as a decrease, in the formation of the complex in the presence of a test compound (relative to what is seen in the absence of the test compound) is indicative of a modulation, e.g., inhibition, of the interaction between the *HDx* protein and its target polypeptide.

Furthermore, the present invention contemplates the use of other homologs of the *HDx* polypeptides or bioactive fragments thereof to generate similar assay formats. In one embodiment, the drug screening assay can be derived with a fungal homolog of an *HDx* protein, such as RPD3, in order to identify agents which inhibit histone deacetylation in a yeast cell.

Yet another aspect of the present invention concerns a method for modulating one or more of growth, differentiation, or survival of a mammalian cell by modulating *HDx* bioactivity, e.g., by inhibiting the deacetylase activity of *HDx* proteins, or disrupting certain protein-protein interactions. In general, whether carried out *in vivo*, *in vitro*, or *in situ*, the method comprises treating the cell with an effective amount of an *HDx* therapeutic so as to alter, relative to the cell in the absence of treatment, at least one of (i) rate of growth, (ii) differentiation, or (iii) survival of the cell. Accordingly, the method can be carried out with *HDx* therapeutics such as peptide and peptidomimetics or other molecules identified in the above-referenced drug screens which antagonize the effects of a naturally-occurring *HDx* protein on said cell. Other *HDx* therapeutics include antisense constructs for inhibiting expression of *HDx* proteins, and dominant negative mutants of *HDx* proteins which competitively inhibit protein-substrate and/or protein-protein interactions upstream and downstream of the wild-type *HDx* protein.

In an exemplary embodiment the subject method is used to treat tumor cells by antagonizing *HDx* activity and blocking cell cycle progression. In one embodiment, the subject method includes the treatment of testicular cells, so as to modulate spermatogenesis. In another embodiment, the subject method is used to modulate osteogenesis, comprising the treatment of osteogenic cells with an *HDx* polypeptide. Likewise, where the treated cell is a chondrogenic cell, the present method is used to modulate chondrogenesis. In still another embodiment, *HDx* polypeptides can be used to modulate the differentiation of progenitor cells, e.g., the method can be used to cause differentiation of a

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hematopoietic cells, neuronal cells, or other stem/progenitor cell populations, to maintain a that cell in a differentiated state, and/or to enhance the survival of a differentiated cell, e.g., to prevent apoptosis or other forms of cell death.

5 In addition to such *HDx* therapeutic uses, anti-fungal agents developed with such screening assays as described herein can be used, for example, as preservatives in foodstuff, feed supplement for promoting weight gain in livestock, or in disinfectant formulations for treatment of non-living matter, e.g., for decontaminating hospital equipment and rooms. In similar fashion, assays provided herein will permit selection of deacetylase inhibitors which discriminate between the human and insect deacetylase  
10 enzymes. Accordingly, the present invention expressly contemplates the use and formulations of the deacetylase inhibitors in insecticides, such as for use in management of insects like the fruit fly. Moreover, certain of the inhibitors can be selected on the basis of inhibitory specificity for plant *HDx*-related activities relative to the mammalian enzymes. Thus, the present invention specifically contemplates formulations of  
15 deacetylase inhibitors for agricultural applications, such as in the form of a defoliant or the like.

The present method is applicable, for example, to cell culture technique, such as in the culturing of hematopoietic cells and other cells whose survival or differentiative state is dependent on *HDx* function. Moreover, *HDx* agonists and antagonists can be  
20 used for therapeutic intervention, such as to enhance survival and maintenance of cells, as well as to influence organogenic pathways, such as tissue patterning and other differentiation processes. In an exemplary embodiment, the method is practiced for modulating, in an animal, cell growth, cell differentiation or cell survival, and comprises administering a therapeutically effective amount of an *HDx* polypeptide to alter, relative  
25 the absence of *HDx* treatment, at least one of (i) rate of growth, (ii) differentiation, or (iii) survival of one or more cell-types in the animal.

Another aspect of the present invention provides a method of determining if a subject, e.g. a human patient, is at risk for a disorder characterized by unwanted cell proliferation or aberrant control of differentiation. The method includes detecting, in a  
30 tissue of the subject, the presence or absence of a genetic lesion characterized by at least one of (i) a mutation of a gene encoding an *HDx* protein, e.g. represented in one of SEQ ID Nos: 1-4, or a homolog thereof; or (ii) the mis-expression of an *HDx* gene. In preferred embodiments, detecting the genetic lesion includes ascertaining the existence of at least one of: a deletion of one or more nucleotides from an *HDx* gene; an addition of  
35 one or more nucleotides to the gene, a substitution of one or more nucleotides of the



gene, a gross chromosomal rearrangement of the gene; an alteration in the level of a messenger RNA transcript of the gene; the presence of a non-wild type splicing pattern of a messenger RNA transcript of the gene; or a non-wild type level of the protein.

For example, detecting the genetic lesion can include (i) providing a probe/primer  
5 including an oligonucleotide containing a region of nucleotide sequence which hybridizes to a sense or antisense sequence of an *HDx* gene, e.g. a nucleic acid represented in one of SEQ ID Nos: 1-4, or naturally occurring mutants thereof, or 5' or 3' flanking sequences naturally associated with the *HDx* gene; (ii) exposing the probe/primer to nucleic acid of the tissue; and (iii) detecting, by hybridization of the probe/primer to the nucleic acid, the  
10 presence or absence of the genetic lesion; e.g. wherein detecting the lesion comprises utilizing the probe/primer to determine the nucleotide sequence of the *HDx* gene and, optionally, of the flanking nucleic acid sequences. For instance, the probe/primer can be employed in a polymerase chain reaction (PCR) or in a ligation chain reaction (LCR). In alternate embodiments, the level of an *HDx* protein is detected in an immunoassay using  
15 an antibody which is specifically immunoreactive with the *HDx* protein.

In another aspect, the invention provides compounds useful for inhibition of *HDxs*. In a preferred embodiment, an *HDx* inhibitor compound of the invention can be represented by the formula A-B-C, in which A is a specificity element for selective binding to an *HDx*, B is a linker element, and C is an electrophilic moiety capable of  
20 reacting with a nucleophilic moiety of an *HDx*, with the proviso that the compound is not butyrate, trapoxin, or trichostatin.

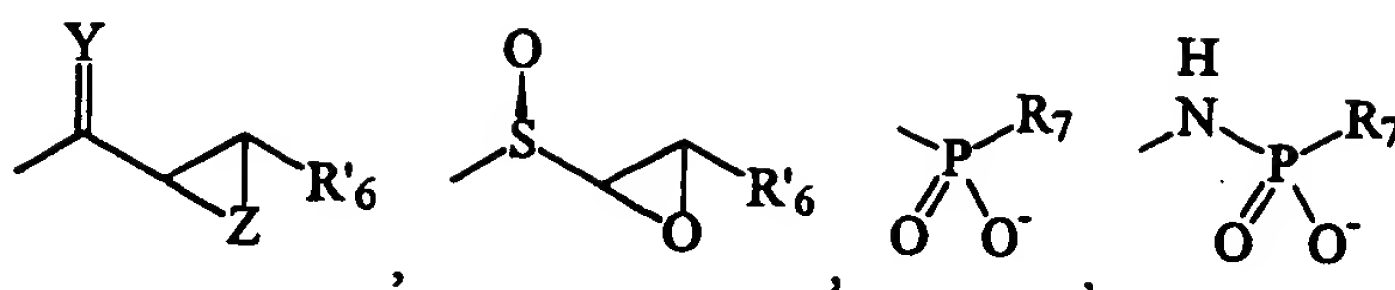
For instance, in one embodiment, there is provided a composition for inhibiting a histone deacetylase comprising a compound represented by the general formula A-B-C, wherein

25 A is selected from the group consisting of cycloalkyls, unsubstituted and substituted aryls, heterocyclyls, amino acyls, and cyclopeptides;

B is selected from the group consisting of substituted and unsubstituted C<sub>4</sub>-C<sub>8</sub> alkylidenes, C<sub>4</sub>-C<sub>8</sub> alkenylidenes, C<sub>4</sub>-C<sub>8</sub> alkynylidenes, and -(D-E-F)-, in which D and F are, independently, absent or represent a C<sub>2</sub>-C<sub>7</sub> alkylidene, a C<sub>2</sub>-C<sub>7</sub> alkenylidene or a C<sub>2</sub>-  
30 C<sub>7</sub> alkynylidene, and E represents O, S, or NR', in which R' represents H, a lower alkyl, a lower alkenyl, a lower alkynyl, an aralkyl, aryl, or a heterocyclyl; and

C is selected from the group consisting of

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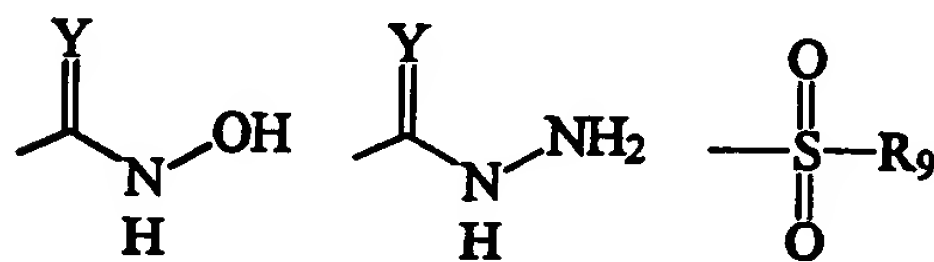
and a boronic acid; in which Z represents O, S, or NR<sub>5</sub>, and Y; R<sub>5</sub> represents a hydrogen, an alkyl, an alkoxy, an aryloxy, an alkylsulfonyl, an arylsulfonyl or an aryl; R'<sub>6</sub> represents hydrogen, an alkyl, an alkenyl, an alkynyl or an aryl; and R<sub>7</sub> represents a hydrogen, an alkyl, an aryl, an alkoxy, an aryloxy, an amino, a hydroxylamino, an alkoxyamino or a halogen; with the proviso that the compound is not trapoxin.

In another preferred embodiment, the compound represented by the general formula A-B-C, wherein

A is selected from the group consisting of cycloalkyls, unsubstituted and substituted aryls, heterocyclyls, amino acyls, and cyclopeptides;

B is selected from the group consisting of substituted and unsubstituted C<sub>4</sub>-C<sub>8</sub> alkylidenes, C<sub>4</sub>-C<sub>8</sub> alkenylidenes, C<sub>4</sub>-C<sub>8</sub> alkynylidenes, and -(D-E-F)-, in which D and F are, independently, absent or represent C<sub>2</sub>-C<sub>7</sub> alkylidenes, C<sub>2</sub>-C<sub>7</sub> alkenylidenes or C<sub>2</sub>-C<sub>7</sub> alkynylidenes, and E represents O, S, or NR', in which R' represents H, a lower alkyl, a lower alkenyl, a lower alkynyl, an aralkyl, an aryl, or a heterocyclyl; and

C is selected from the group consisting of



in which R<sub>9</sub> represents a hydrogen, an alkyl, an aryl, a hydroxyl, an alkoxy, an aryloxy or an amino,

with the proviso that the inhibitor compound is not trichostatin.

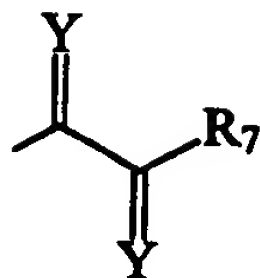
In still another preferred embodiment, the compound is represented by the general formula A-B-C, wherein

A is selected from the group consisting of cycloalkyls, unsubstituted and substituted aryls, heterocyclyls, amino acyls, and cyclopeptides;

B is selected from the group consisting of substituted and unsubstituted C<sub>4</sub>-C<sub>8</sub> alkylidenes, C<sub>4</sub>-C<sub>8</sub> alkenylidenes, C<sub>4</sub>-C<sub>8</sub> alkynylidenes, and -(D-E-F)-, in which D and F are, independently, absent or a C<sub>2</sub>-C<sub>7</sub> alkylidene, a C<sub>2</sub>-C<sub>7</sub> alkenylidene, or a C<sub>2</sub>-C<sub>7</sub> alkynylidene, and E represents O, S, or NR', in which R' is H, lower alkyl, lower alkenyl, lower alkynyl, aralkyl, aryl, or heterocyclyl; and



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C represents ; in which Y is O or S, and R<sub>7</sub> represents a hydrogen, an alkyl, an aryl, an alkoxy, an aryloxy, an amino, a hydroxylamino, an alkoxyamino or a halogen.

The present invention also contemplates pharmaceutical preparations of such compounds, e.g., in an amount effective for inhibiting proliferation of a cell, formulated in a pharmaceutically acceptable diluent.

Moreover, such compounds can be used for modulating one or more of growth, differentiation, or survival of a mammalian cell responsive to HDx-mediated histone deacetylation, by treating the cell with an effective amount of the deacetylase inhibitor so as to modulate the deacetylase activity and alter, relative to the cell in the absence of the agent, at least one of (i) the rate of growth, (ii) the differentiation state, or (iii) the rate of survival of the cell.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

### *Brief Description of the Drawings*

5        Figure 1A illustrates the chemical structures of trapoxin and trichostatin, natural products that inhibit the enzymatic deacetylation of lysine residues near the NH<sub>2</sub>-terminus of histones. The epoxyketone side chain of trapoxin is approximately isosteric with N-acetyl lysine and likely alkylates an active site nucleophile.

10        Figure 1B illustrates the copurification of trapoxin binding and histone deacetylase activities. Nuclear proteins from bovine thymus were precipitated with ammonium sulfate and fractionated on a Mono Q column. Trapoxin *binding was assayed by charcoal precipitation* with [<sup>3</sup>H]trapoxin. For the histone deacetylase assay, a peptide corresponding to bovine histone H4 (1-24) was synthesized. The peptide was chemically acetylated with sodium [<sup>3</sup>H]acetate (5.3 Ci/mmol, New England  
15    Nuclear)/BOP reagent (Aldrich) and purified by reverse phase HPLC. Two microliters of [<sup>3</sup>H]peptide (~40,000dpm) were used per 200 µl assay. After incubation at 37°C for one hour, the reaction was quenched with 1 M HCl/0.16 M acetic acid (50 µl). Released [<sup>3</sup>H]acetic acid was extracted with 600 µl of ethyl acetate and quantified by scintillation counting. Pretreatment of crude or partially purified enzyme with trapoxin or trichostatin  
20    (20nM) for 30 min. at 4°C abolished deacetylase activity. A<sub>280</sub> = absorbance at 280 nm.

Figure 2A shows the synthesis of K-trap and the K-trap affinity matrix. K-trap contains a protected lysine residue in place of the phenylalanine at position two in trapoxin. Alloc = allyloxycarbonyl.

25        Figure 2B is a silver stained gel showing bovine and human trapoxin binding proteins. Proteins bound to the K-trap affinity matrix in the *presence* or *absence* of trapoxin or trichostatin were eluted by boiling in SDS loading buffer and analyzed by SDS-PAGE (9% gel). Nuclear proteins from human Jurkat T cells were prepared identically to those from bovine thymus (Figure 1B). Molecular size standards (in kilodaltons) are indicated to the right.

30        Figure 3A is the predicted amino acid sequence of human *HD1*. An in-frame stop codon was found upstream of the starting methionine. Regions equivalent to microsequenced tryptic peptides from the purified bovine protein are boxed. Underlined amino acids 319-334 and 467-482 denote the sequences of synthetic peptides that were conjugated to KLH and used to generate polyclonal antisera. Abbreviations for the

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amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Figure 3B is a protein immunoblot analogous to the silver stained gel in Figure 2B, showing the relationship between bovine p46-p49 and human p55 (top panels) and confirming the identity of p50 (bovine and human) as RbAp48 (bottom panels). Proteins eluted from the K-trap affinity matrix (Figure 2) were separated by SDS-PAGE and transferred to Immobilon-P (Millipore). Blots were probed with polyclonal anti-*HD1* (319-336) or monoclonal anti-RbAp48 and bound antibodies were detected with enhanced chemiluminescence (Amersham).

Figure 4A is an immunoprecipitation of endogenous histone deacetylase activity with affinity purified anti-*HD1*(467-482) antibodies. Anti-*HD1*(467-482) immunoprecipitates from equivalent amounts of Jurkat nuclear extract (1 mg nuclear protein supplemented with 0.5 M NaCl, 1% BSA, and 0.1% NP-40) were isolated in the presence or absence of *HD1*(467-482) peptide competitor. After resuspending the immunoprecipitates in *HDx* buffer [20 mM tris (pH 8), 150 mM NaCl, 10% glycerol], inhibitors were added as indicated, and histone deacetylase activity was measured as described in Figure 1A.

Figure 4B shows the coprecipitation of *HD1* and RbAp48, as detected by protein immunoblot analysis.

Figure 4C demonstrates the histone deacetylase activity of recombinant *HD1*-F. Tag Jurkat cells (Clipstone et al. (1992) *Nature* 357, 695-7) were transfected with pFJ5 (vector alone) or pBJ5/*HD1*-F (encoding COOH-terminal FLAG epitope tagged *HD1*) by electroporation and detergent lysates were prepared [0.5% Triton X-100, 50 mM tris (pH 8), 100 mM NaCl, 10% glycerol]. Anti-FLAG antibodies conjugated to agarose beads (IBI) were used to immunoprecipitate recombinant *HD1* in the presence or absence of FLAG peptide competitor, and histone deacetylase activity was measured as described above.

Figure 4D shows the interaction between recombinant *HD1*-F and the K-trap affinity matrix. Lysates from Jurkat cells transfected with pBJ5/*HD1*-F were incubated with the K-trap affinity matrix in the presence or absence of inhibitors. Immunoblots of the eluted proteins were probed with the anti-FLAG M2 monoclonal antibody (IBI).

Figures 5A and 5B are sequence alignments for various *HDx* and *HDx*-related cDNAs and proteins, respectively.

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Figure 6 depicts exemplary specificity elements (A), linker elements (B), and electrophilic moieties (C) for generating compounds which are capable of reacting with a nucleophilic moiety of an HDx protein.

Figure 7 illustrates an exemplary synthesis of trichostatin analogs.

5     Figures 8A-8C illustrate a synthesis of tritiated Trapoxin B.

Figures 9A-9C depict a synthesis of the K-trap and K-trap affinity matrix .

10     Figures 10A-10B: *mSin3A* is present in cells as a large stable multiprotein complex. Nuclear lysates were prepared from U937 cells metabolically labeled with [<sup>35</sup>S]-methionine and low stringency immunoprecipitations performed with antiserum specific for mSin3A. "+ block" shows proteins immunoprecipitated when the anti-mSin3A was preincubated with purified GST-PAH2 (A). In (B), low stringency mSin3A immunoprecipitates were washed for an additional 60 minutes using the salt and detergent conditions indicated at the top of the Figure. In (A) and (B), the immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Apparent  
15     molecular weight of the coprecipitating proteins and the sizes of the molecular weight markers are given in kilodaltons.

20     Figures 11A-D: *mSin3A* and *EMACI* associate in vivo. Immunoprecipitations were performed using nuclear extracts from [<sup>35</sup>S]-methionine labeled U937 cells. (A) The left lane shows proteins from an anti-mSin3A immunoprecipitate. The right lane shows proteins eluted from an anti-mSin3A immunoprecipitate and reprecipitated with anti-serum specific for HD1. In (B) and (C), low stringency immunoprecipitations were performed using antiserum specific for the carboxy-terminus of HD1. "+ block" indicates that the HD1 antiserum was preincubated with the immunizing peptide. In (C), proteins immunoprecipitated with anti-mS3A are shown for reference, proteins eluted from a low  
25     stringency anti-HD1 immunoprecipitate and reprecipitated with anti-mSin3A are shown in the right most lane. In (A), (B), and (C), autoradiographs of SDS-PAGE gels are shown. Apparent molecular weight of the coprecipitating, proteins and the sizes of the molecular weight markers are given in kilodaltons. In (D), in vitro histone deacetylase activity in anti-mSin3A immunoprecipitates is shown. Human Jurkat cell extracts (12  
30     mg) were immunoprecipitated using anti-mSin3A polyclonal antibodies, "+block" indicates that the anti-mSin3A antibody was preincubated with GST-PAH2, "+10 nM trapoxin" indicates that the immunoprecipitated proteins were pretreated with 10 nM trapoxin for 30 minutes at 4°C prior to being assayed for histone deacetylase activity.

Figures 12A-C: *RbAp48 is associated with mSin3A in vivo and recombinant HD1, RbAp48 and mSin3A copurify from insect cell extracts.* (A) TAg Jurkat cell lysates were immunoprecipitated using antibodies specific to C-terminal portion of HD1 (left) or antibodies specific to PAH2 of mSin3A (right). Parallel immunoprecipitations were blocked as described in figure 11. Immunopurified proteins were analyzed by SDS-PAGE and immunoblotted with (x-RbAp48 monoclonal antibody 12B1. (B & C) Equal amounts of baculovirus coinfecting Sf9 cell extracts were affinity purified using Ni<sup>2+</sup>-agarose "Ni" or x-FLAG-M2-agarose "F". Purified recombinant proteins were analyzed by SDS-PAGE, transferred to Immobilon-P (Millipore) and immunoblotted with FLAG to detect HD1-F (B) or (x-Flu (12CA.5) to detect p48-HA (C). We observe a reduction in expression of HD1-F and p48-HA when coexpressed with mSin3A.

Figures 13A-C: *Trapoxin reverses transcriptional repression by mSin3A.* (A) The structure of the minimal reporter gene derived from the myelomonocytic growth factor gene and the expression vectors. Mad(Pro)N35GALVPI6 has leucine at position 12 and alanine at position 16 mutated to proline as indicated. These point mutations prevent association between mSin3A and Mad (Ayer et al., 1995). The transcriptional activity of MadN35GALVP16 and Mad(Pro)N35GALVP16 was determined by measuring luciferase activity (Relative Light Units, RLU) of transfected 293 cells following an 8 hour treatment with 0 (solid bars) or 10 nM trapoxin (striped bars) (B). To control for differences in transfection efficiency, the RLU values were normalized to the  $\beta$ -galactosidase activity produced by a cotransfected CMV- $\beta$ GAL construct. Shown is data from representative experiment and the error is reported as the standard error of the mean (s.e.m). This experiment has been done a minimum of five times in triplicate with similar results. An 8 hour treatment of 293 cells with 10 nM trapoxin is within the linear range of the response of the reporter gene. Furthermore, trapoxin treatment did not prevent association between mSin3A and HD1 (data not shown). (C), trapoxin inhibits histone deacetylase activity of human 293 cells in vivo.  $2 \times 10^8$  cells were cultured for 8 hours in the absence, "O", or in the presence of 10 nM trapoxin. Cells were harvested and crude extracts from approximately  $1 \times 10^7$  cells (solid bars) or anti-HD1 immunoprecipitations of extracts from approximately  $4 \times 10^7$  cells (white bars) were assayed for histone deacetylase activity in vitro.



### *Detailed Description of the Invention*

The positioning of nucleosomes relative to particular regulatory elements in genomic DNA has emerged as a mechanism for managing the association of sequence-specific DNA-binding proteins with promoters, enhancers and other transcriptional regulatory sequences. Two modifications to nucleosomes have been observed to influence the association of DNA-binding proteins with chromatin. Depletion of histones H2A/H2B from the nucleosome facilitates the binding of RNA polymerase II (Baer et al. (1983) *Nature* 301:482-488) and TFIIIA (Hayes et al. (1992) *PNAS* 89:1229-1233). Likewise, acetylation of the core histones apparently destabilizes the nucleosome and is thought to modulate the accessibility of transcription factors to their respective enhancer and promoter elements (Oliva et al. (1990) *Nuc Acid Res* 18:2739-2747; and Walker et al. (1990) *J Biol Chem* 265:5622-5746). In both cases, overall histone-DNA contacts are altered.

In one aspect, the present invention concerns the discovery of a family of genes in mammals, the gene products of which are referred to herein as "histone deacetylases" or "*HDx*'s". Experimental evidence indicates a functional role for the *HDx* gene products as catalysts of the deacetylation of histones in mammalian cells, and accordingly play a role in determining tissue fate and maintenance. For instance, the results provided below indicate that proteins encoded by the *HDx* genes may participate, under various circumstances, in the control of proliferation, differentiation and cell death.

The family of *HDx* gene apparently encode at least three different sub-families, e.g., paralogs, and have been identified from the cells of various mammals. The *HDx* proteins were first isolated from bovine thymus nuclei by use of a binding assay which exploited the ability of trapoxin, an inhibitor of histone deacetylase activity, to isolate proteins which co-purified with a histone acetylase activity. The partial identity of the isolated proteins were determined by peptide microsequencing, and primers based on the peptide sequences were used to clone human cDNAs from a T cell library. One of the *HDx* gene products described below is referred to herein as *HD1*, and is represented in SEQ ID No. 1 (nucleotide) and SEQ ID No. 5 (amino acid).

A search of expressed sequence tag (EST) libraries turned up partial sequences for human *HDx* transcripts, and revealed the existence of at least two other human *HDx* genes related to *HD1*, these other paralogs referred to herein as *HD2* and *HD3*. Nucleotide and amino acid sequences for partial clones of other human *HDx* homologs are provided by SEQ ID Nos. 2-4 and 6-8, respectively.

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Analysis of the *HDx* sequences indicated no obvious similarities with any previously identified domains or motifs. However, the fact that each full-length clone lacks a signal sequence, along with the observation that proteins can be detected in the nucleus, indicates that the *HDx* genes encode intracellular proteins.

- 5 Careful inspection of the *HDx* clones suggests at least two novel motifs, one or both of which may be characteristic of at least subfamilies of the mammalian *HDx* family. The first apparently conserved structural element of the *HDx* family occurs in the N-terminal portion of the molecule, and is designated herein as the "v motif". With reference to human *HD1*, the v motif corresponds to amino acid residues Asp130-  
10 Phe198. By alignment of the human *HDx* sequences, the element is represented by the consensus sequence:

DXXXNXXGGLHHAKKXEASGFCYXNDIVXXIXELLXYHXR VXYIDXDXXHHGDGXEEAFYXTDR  
VMTXSF, (SEQ ID No. 12)

more preferably by the consensus sequence:

- 15 DIAX<sub>1</sub>NWAGGLHHAKKX<sub>2</sub>EASGFCYVNDIVX<sub>3</sub>X<sub>4</sub>ILELLKYHX<sub>5</sub>RVLYIDIDIHHGDGX<sub>6</sub>EEAFYX  
7TD-RVMTVSF (SEQ ID No. 13)

wherein each of X<sub>n</sub> represents any single amino acid, though more preferably represents an amino acid residue in the corresponding human *HDx* sequences of the appended sequence listing.

- 20 A second motif, herein designated the  $\chi$  motif is represented by the consensus sequence:

CVXXXXXFXXPXXXXGGGGYTXRNVARXWXXET (SEQ ID No. 14)

more preferably by the consensus sequence:

CVEX<sub>8</sub>VKX<sub>9</sub>FNX<sub>10</sub>PLLX<sub>11</sub>LGGGGYTX<sub>12</sub>RNVARCWTYET (SEQ ID No: 15)

- 25 wherein each of X<sub>n</sub> represents any single amino acid, though more preferably represents an amino acid residue in the corresponding human *HDx* sequences of the appended sequence listing. The  $\chi$  motif can be found in the human *HD1* sequence at C284-Thr316.

- The family of *HDx* proteins apparently ranges in size from about 40kd to about 60kd for the unmodified polypeptide chain. For instance, the bovine *HD1* protein  
30 migrates on an SDS-PAGE (9%) gel with an apparent molecular weight of 46kD. The human *HD1* amino acid sequence predicts a molecular weight for the polypeptide chain of 55kD.



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Accordingly, certain aspects of the present invention relate to nucleic acids encoding *HDx* proteins, the *HDx* proteins themselves, antibodies immunoreactive with *HDx* proteins, and preparations of such compositions. Moreover, the present invention provides diagnostic and therapeutic assays and reagents for detecting and treating disorders involving, for example, aberrant expression (or loss thereof) of *HDx* homologs. In addition, drug discovery assays are provided for identifying agents which can modulate the biological function of *HDx* proteins, such as by altering the binding of *HDx* molecules to either proteins or nucleic acids. Such agents can be useful therapeutically to alter the growth and/or differentiation of a cell. Other aspects of the invention are described below or will be apparent to those skilled in the art in light of the present disclosure.

Analysis of the human *HDx* sequences, while not revealing any obvious similarities to known domains or motifs, did indicate similarities with previously identified proteins from both *Saccharomyces cerevisiae* and *Xenopus laevis*. Those genes, RPD3 (SEQ ID No. 9) and Xe-RPD3 (SEQ ID No. 10), respectively, had not previously been ascribed any specific function. However, based on our observations for the function of *HDI*, it is now apparent that each of these other proteins are also deacetylases, and represent potential therapeutic targets. Accordingly, drug discovery assays are provided for identifying agents which can modulate the biological function of "*HDx*-related" proteins, such as RPD3 homologs, by altering the enzymatic activity of the deacetylase, or its binding to other cellular components including homologs of RbAp48 (described *infra*). Such agents can be useful therapeutically to alter the growth and/or differentiation of non-human cells, such as in the treatment of mycotic infections, or as additives to livestock feed, e.g., to promote weight gain, or as topical antiseptics for sterilizing medical equipment.

In addition we isolated another bovine protein having an approximate molecular size of 50kD which apparently binds *HDx* proteins isolated by the trapoxin matrix, and microsequencing of that protein demonstrated that it was related to the protein referred to in the art as RbAp48 (Qian et al. (1993) *Nature* 364:648; SEQ ID No. 11). RbAp48 was originally identified as a protein that binds to the retinoblastoma (Rb) gene product. The retinoblastoma (RB) gene product plays a role in tumor suppression (Weinberg, R.A., (Sept 1988) *Scientific Amer.* pp 44-51; Hansen et al. (1988) *Trends Genet* 4:125-128). The role of RB as a tumor-suppressor protein in cell-cycle control is believed to be similar to that of another tumor-suppressor, p53 (Green (1989) *Cell* 56:1-3; Mowat et al (1985) *Nature* 314:633-636). Inactivation or mutation of the second RB allele in one of the somatic cells of these susceptible individuals appears to be the molecular event that

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leads to tumor formation (Caveneee et al. (1983) *Nature* 305:799-784; Friend et al. (1987) *PNAS* 84:9059-9063).

The growth suppression function of the retinoblastoma protein is thought to be mediated by Rb binding to cellular proteins. RbAp48 is one of the major proteins that  
5 binds to a putative functional domain at the carboxy terminus of the Rb protein. Complex formation between RbAp48 and Rb occurs *in vitro* and *in vivo*, and apparently involves direct interaction between the proteins. Like Rb, RbAp48 is a ubiquitously expressed nuclear protein. RbAp48 share sequence homology with *MSI1*, a negative  
10 regulator of the Ras-cyclic AMP pathway in the yeast *Saccharomyces cerevisiae*. Furthermore, like *MSI1*, human RbAp48 suppresses the heat-shock sensitivity of the yeast *iral* strains and *RAS2Val19* strains. Interaction with RbAp48 may be one of the mechanisms for suppression of growth mediated by Rb. Accordingly, the interaction of RbAp48 with *HDx* proteins further implicates the *HDx* proteins in cell-cycle regulation.

The RbAp48 interaction with *HDx* and *HDx*-related proteins represents yet  
15 another therapeutic target. Accordingly, drug discovery assays are provided for identifying agents which can modulate the interaction of RbAp48 proteins and the like with *HDx*-related proteins. Such assays can be derived to detect the ability of a test agent to alter protein-protein contacts, or to alter the enzymatic activity of the deacetylase in complexes including an RbAp48 protein (e.g., were such complexes  
20 allosterically modulate the *HDx* enzymatic activity). As above, such agents can be useful therapeutically to alter the growth and/or differentiation of cells.

Members of the Mad family of BHLHZip proteins heterodimerize with Max to repress transcription in a sequence-specific manner. Transcriptional repression by Mad:Max heterodimers is mediated by ternary complex formation with either of the  
25 corepressors mSin3A or mSin3B. Example 3 demonstrates that Sin3 proteins are an *in vivo* component of large, heterogeneous multiprotein complexes and is tightly and specifically associated with at least seven polypeptides. Two of the Sin3-associated proteins, p50 and p55, are members of the histone deacetylase family described herein. Sin3 immunocomplexes possess histone deacetylase activity that is sensitive to the  
30 specific deacetylase inhibitor trapoxin. Sin3 targeted repression of a reporter gene is reduced by trapoxin treatment, suggesting that histone deacetylation mediates transcriptional repression through Mad-Max-Sin3A multimeric complexes.

The Sin3 interaction with *HDx* and *HDx*-related proteins represents still another therapeutic target. Thus, in one aspect of the present invention there is provided drug

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discovery assays for identifying agents which can modulate the interaction of Sin3 proteins and the like with *HDx*-related proteins. Such assays can be derived to detect the ability of a test agent to alter protein-protein contacts, or to alter the enzymatic activity of the deacetylase in complexes including Sin3 or other transcriptional regulatory proteins. As above, such agents can be useful therapeutically to alter the growth and/or differentiation of cells.

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding one of the *HDx* polypeptides of the present invention, including both exon and (optionally) intron sequences. A "recombinant gene" refers to nucleic acid encoding an *HDx* polypeptide and comprising *HDx*-encoding exon sequences, though it may optionally include intron sequences which are either derived from a chromosomal *HDx* gene or from an unrelated chromosomal gene. Exemplary recombinant genes encoding the subject *HDx* polypeptide are represented in the appended Sequence Listing. The term "intron" refers to a DNA sequence present in a given *HDx* gene which is not translated into protein and is generally found between exons.

As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a recombinant form of an *HDx* polypeptide or, where anti-sense expression occurs from the transferred gene, the expression of a naturally-occurring form of the *HDx* protein is disrupted.

As used herein, the term "specifically hybridizes" refers to the ability of the probe/primer of the invention to hybridize to at least 15 consecutive nucleotides of an *HDx* gene, such as an *HDx* sequence designated in one of SEQ ID Nos: 1-4, or a sequence complementary thereto, or naturally occurring mutants thereof, such that it has

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less than 15%, preferably less than 10%, and more preferably less than 5% background hybridization to a cellular nucleic acid (e.g., mRNA or genomic DNA) encoding a protein other than an *HDx* protein, as defined herein. In preferred embodiments, the oligonucleotide probe specifically detects only one of the subject *HDx* paralogs, e.g.,  
5 does not substantially hybridize to transcripts for other *HDx* homologs in the same species.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication.  
10 Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which,  
15 in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

20 "Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked. In preferred embodiments, transcription of one of the recombinant *HDx* genes is under the control of a promoter sequence (or other  
25 transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring forms of *HDx* genes.

30 As used herein, the term "tissue-specific promoter" means a DNA sequence that serves as a promoter, i.e., regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in specific cells of a tissue, such as cells of hepatic, pancreatic, neuronal or hematopoietic origin. The term also covers so-called "leaky" promoters, which regulate expression of a

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selected DNA primarily in one tissue, but can cause at least low level expression in other tissues as well.

As used herein, a "transgenic animal" is any animal, preferably a non-human mammal, bird or an amphibian, in which one or more of the cells of the animal contain  
5 heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or *in vitro*  
10 fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. In the typical transgenic animals described herein, the transgene causes cells to express a recombinant form of one of the HDx proteins, e.g. either agonistic or antagonistic forms. However, transgenic animals in which the recombinant HDx gene is  
15 silent are also contemplated, as for example, the FLP or CRE recombinase dependent constructs described below. Moreover, "transgenic animal" also includes those recombinant animals in which gene disruption of one or more HDx genes is caused by human intervention, including both recombination and antisense techniques.

The "non-human animals" of the invention include vertebrates such as rodents,  
20 non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, etc. Preferred non-human animals are selected from the rodent family including rat and mouse, most preferably mouse, though transgenic amphibians, such as members of the *Xenopus* genus, and transgenic chickens can also provide important tools for understanding and identifying agents which can affect, for example, embryogenesis and tissue formation.  
25 The invention also contemplates transgenic insects, including those of the genus *Drosophila*, such as *D. melanogaster*. The term "chimeric animal" is used herein to refer to animals in which the recombinant gene is found, or in which the recombinant is expressed in some but not all cells of the animal. The term "tissue-specific chimeric animal" indicates that one of the recombinant HDx genes is present and/or expressed or  
30 disrupted in some tissues but not others.

As used herein, the term "transgene" means a nucleic acid sequence (encoding, e.g., one of the HDx polypeptides, or pending an antisense transcript thereto), which is partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell  
35 into which it is introduced, but which is designed to be inserted, or is inserted, into the



animal's genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene can include one or more transcriptional regulatory sequences and any other nucleic acid, such as introns, that may be necessary for optimal  
5 expression of a selected nucleic acid.

As is well known, genes for a particular polypeptide may exist in single or multiple copies within the genome of an individual. Such duplicate genes may be identical or may have certain modifications, including nucleotide substitutions, additions or deletions, which all still code for polypeptides having substantially the same activity. The  
10 term "DNA sequence encoding an *HDx* polypeptide" may thus refer to one or more genes within a particular individual. Moreover, certain differences in nucleotide sequences may exist between individuals of the same species, which are called alleles. Such allelic differences may or may not result in differences in amino acid sequence of the encoded polypeptide yet still encode a protein with the same biological activity.

15 "Homology" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology between sequences is a function of  
20 the number of matching or homologous positions shared by the sequences. An "unrelated" or "non-homologous" sequence shares less than 40 percent identity, though preferably less than 25 percent identity, with one of the *HDx* sequences of the present invention.

As used herein, an "*HDx*-related" protein refers to the *HDx* proteins described  
25 herein, and other human homologs of those *HDx* sequences, as well as orthologs and paralogs (homologs) of the *HDx* proteins in other species, ranging from yeast to other mammals, e.g., homologous histone deacetylase. The term "ortholog" refers to genes or proteins which are homologs via speciation, e.g., closely related and assumed to have common descent based on structural and functional considerations. Orthologous proteins  
30 function as recognizably the same activity in different species. The term "paralog" refers to genes or proteins which are homologs via gene duplication, e.g., duplicated variants of a gene within a genome. See also, Fritch, WM (1970) *Syst Zool* 19:99-113.

"Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to

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the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

5       A "chimeric protein" or "fusion protein" is a fusion of a first amino acid sequence encoding one of the subject *HDx* polypeptides with a second amino acid sequence defining a domain (e.g. polypeptide portion) foreign to and not substantially homologous with any domain of one of the *HDx* proteins. A chimeric protein may present a foreign domain which is found (albeit in a different protein) in an organism which also expresses  
10 the first protein, or it may be an "interspecies", "intergenic", etc. fusion of protein structures expressed by different kinds of organisms. In general, a fusion protein can be represented by the general formula *X-HDx-Y*, wherein *HDx* represents a portion of the protein which is derived from one of the *HDx* proteins, and *X* and *Y* are, independently, absent or represent amino acid sequences which are not related to one of the *HDx*  
15 sequences in an organism.

The term "isolated" as also used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs, or RNAs, respectively, that are present in the natural source of the macromolecule. For example, an isolated nucleic acid encoding one of the subject *HDx* polypeptides preferably includes no more  
20 than 10 kilobases (kb) of nucleic acid sequence which naturally immediately flanks the *HDx* gene in genomic DNA, more preferably no more than 5kb of such naturally occurring flanking sequences, and most preferably less than 1.5kb of such naturally occurring flanking sequence. The term isolated as used herein also refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture  
25 medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state.

As described below, one aspect of the invention pertains to isolated nucleic acids  
30 comprising nucleotide sequences encoding *HDx* polypeptides, and/or equivalents of such nucleic acids. The term nucleic acid as used herein is intended to include fragments as equivalents. The term equivalent is understood to include nucleotide sequences encoding functionally equivalent *HDx* polypeptides or functionally equivalent peptides having an activity of an *HDx* protein such as described herein. Equivalent nucleotide sequences will  
35 include sequences that differ by one or more nucleotide substitutions, additions or



deletions, such as allelic variants; and will, therefore, include sequences that differ from the nucleotide sequence of the *HDx* cDNA sequences shown in any of SEQ ID Nos:1-4 due to the degeneracy of the genetic code. Equivalents will also include nucleotide sequences that hybridize under stringent conditions (i.e., equivalent to about 20-27°C  
5 below the melting temperature ( $T_m$ ) of the DNA duplex formed in about 1M salt) to the nucleotide sequences represented in one or more of SEQ ID Nos:1-4. In one embodiment, equivalents will further include nucleic acid sequences derived from and evolutionarily related to, a nucleotide sequences shown in any of SEQ ID Nos:1-4.

Moreover, it will be generally appreciated that, under certain circumstances, it  
10 may be advantageous to provide homologs of one of the subject *HDx* polypeptides which function in a limited capacity as one of either an *HDx* agonist (mimetic) or an *HDx* antagonist, in order to promote or inhibit only a subset of the biological activities of the naturally-occurring form of the protein. Thus, specific biological effects can be elicited by treatment with a homolog of limited function, and with fewer side effects relative to  
15 treatment with agonists or antagonists which are directed to all of the biological activities of naturally occurring forms of *HDx* proteins.

Homologs of each of the subject *HDx* proteins can be generated by mutagenesis, such as by discrete point mutation(s), or by truncation. For instance, mutation can give rise to homologs which retain substantially the same, or merely a subset, of the biological  
20 activity of the *HDx* polypeptide from which it was derived. Alternatively, antagonistic forms of the protein can be generated which are able to inhibit the function of the naturally occurring form of the protein, such as by competitively binding to an *HDx* substrate or *HDx* associated protein, as for example competing with wild-type *HDx* in the binding of RbAp48 or a histone. In addition, agonistic forms of the protein may be  
25 generated which are constitutively active, or have an altered  $K_{cat}$  or  $K_m$  for deacetylation reactions. Thus, the *HDx* protein and homologs thereof provided by the subject invention may be either positive or negative regulators of transcription and/or replication.

In general, polypeptides referred to herein as having an activity of an *HDx* protein (e.g., are "bioactive") are defined as polypeptides which include an amino acid sequence  
30 corresponding (e.g., identical or homologous) to all or a portion of the amino acid sequences of an *HDx* proteins shown in any one or more of SEQ ID Nos:5-8 and which mimic or antagonize all or a portion of the biological/biochemical activities of a naturally occurring *HDx* protein. Examples of such biological activity include the ability to modulate proliferation of cells. For example, inhibiting histone deacetylation causes cells  
35 to arrest in G1 and G2 phases of the cell cycle. The biochemical activity associated with

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*HDx* proteins of the present invention can also be characterized in terms of binding to and (optionally) catalyzing the deacetylation of an acetylated histone. Another biochemical property of certain of the subject *HDx* proteins involves binding to other cellular proteins, such as RbAp48 or Sin3A.

- 5 Other biological activities of the subject *HDx* proteins are described herein or will be reasonably apparent to those skilled in the art. According to the present invention, a polypeptide has biological activity if it is a specific agonist or antagonist of a naturally-occurring form of an *HDx* protein.

- 10 Preferred nucleic acids encode an *HDx* polypeptide comprising an amino acid sequence at least 80% homologous, more preferably at least 85% homologous and most preferably at least 88% homologous with an amino acid sequence of a human *HDx*, e.g., such as selected from the group consisting of SEQ ID Nos: 5-8. Nucleic acids which encode polypeptides at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology with an amino acid sequence represented in  
15 one of SEQ ID Nos: 5-8 are of course also within the scope of the invention, as are nucleic acids identical in sequence with any of the enumerated *HDx* sequences of the sequence listing. In one embodiment, the nucleic acid is a cDNA encoding a polypeptide having at least one activity of the subject *HDx* polypeptide.

- 20 In certain preferred embodiments, the invention features a purified or recombinant *HDx* polypeptide having peptide chain with a molecular weight in the range of 40kd to 60kd, even more preferably in the range of 45-50 kd or 53-58kd. It will be understood that certain post-translational modifications, e.g., phosphorylation and the like, can increase the apparent molecular weight of the *HDx* protein relative to the unmodified polypeptide chain, and cleavage of certain sequences, such as pro-sequences, can likewise  
25 decrease the apparent molecular weight.

- In other preferred embodiments, the nucleic acid encodes an *HDx* polypeptide which includes both the  $\nu$  and  $\chi$  motifs, and preferably possess a histone deacetylase activity. For example, preferred *HDx* proteins are represented by the general formula A-( $\nu$  motif)-B-( $\chi$  motif)-C, wherein the  $\nu$  motif is an amino acid sequence represented in  
30 SEQ ID No. 12, more preferably SEQ ID No. 13, the  $\chi$  motif is an amino acid sequence represented in SEQ ID No. 14, more preferably SEQ ID No. 15, and A, B and C represent amino acid sequences which correspond to *HDx* or *HDx*-related proteins.

Still other preferred nucleic acids of the present invention encode an *HDx* polypeptide which includes a polypeptide sequence corresponding to all or a portion of

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amino acid residues of any one of SEQ ID Nos: 5-8, e.g., at least 5, 10, 25, 50 or 100 amino acid residues of that region.

Another aspect of the invention provides a nucleic acid which hybridizes under high or low stringency conditions to the nucleic acid represented by SEQ ID No: 1.

5 Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC

10 at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C.

Nucleic acids, having a sequence that differs from the nucleotide sequences shown in one of SEQ ID Nos: 1-4 due to degeneracy in the genetic code are also within

15 the scope of the invention. Such nucleic acids encode functionally equivalent peptides (i.e., a peptide having a biological activity of an *HDx* polypeptide) but differ in sequence from the sequence shown in the sequence listing due to degeneracy in the genetic code. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC each encode

20 histidine) may result in "silent" mutations which do not affect the amino acid sequence of an *HDx* polypeptide. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject *HDx* polypeptides will exist among, for example, humans. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids

25 encoding polypeptides having an activity of an *HDx* polypeptide may exist among individuals of a given species due to natural allelic variation.

As used herein, an *HDx* gene fragment refers to a nucleic acid having fewer nucleotides than the nucleotide sequence encoding the entire mature form of an *HDx* protein yet which (preferably) encodes a polypeptide which retains some biological

30 activity of the full length protein. Fragment sizes contemplated by the present invention include, for example, 5, 10, 25, 50, 75, 100, or 200 amino acids in length.

As indicated by the examples set out below, *HDx* protein-encoding nucleic acids can be obtained from mRNA present in any of a number of eukaryotic cells. It should also be possible to obtain nucleic acids encoding *HDx* polypeptides of the present

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invention from genomic DNA from both adults and embryos. For example, a gene encoding an *HDx* protein can be cloned from either a cDNA or a genomic library in accordance with protocols described herein, as well as those generally known to persons skilled in the art. A cDNA encoding an *HDx* protein can be obtained by isolating total mRNA from a cell, e.g. a mammalian cell, e.g. a human cell, including embryonic cells. Double stranded cDNAs can then be prepared from the total mRNA, and subsequently inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques. The gene encoding an *HDx* protein can also be cloned using established polymerase chain reaction techniques in accordance with the nucleotide sequence information provided by the invention. The nucleic acid of the invention can be DNA or RNA. A preferred nucleic acid is a cDNA including a nucleotide sequence represented by one of SEQ ID Nos: 1-4.

Another aspect of the invention relates to the use of the isolated nucleic acid in "antisense" therapy. As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotide probes or their derivatives which specifically hybridize (e.g. binds) under cellular conditions, with the cellular mRNA and/or genomic DNA encoding one or more of the subject *HDx* proteins so as to inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes an *HDx* protein. Alternatively, the antisense construct is an oligonucleotide probe which is generated *ex vivo* and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of an *HDx* gene. Such oligonucleotide probes are preferably modified oligonucleotides which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and are therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775), or peptide nucleic acids (PNAs). Additionally, general approaches to constructing oligomers useful in antisense therapy

have been reviewed, for example, by Van der Krol et al. (1988) *Biotechniques* 6:958-976; and Stein et al. (1988) *Cancer Res* 48:2659-2668.

Accordingly, the modified oligomers of the invention are useful in therapeutic, diagnostic, and research contexts. In therapeutic applications, the oligomers are utilized in a manner appropriate for antisense therapy in general. For such therapy, the oligomers of the invention can be formulated for a variety of routes of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remington's *Pharmaceutical Sciences*, Meade Publishing Co., Easton, PA. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the oligomers of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the oligomers may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

Systemic administration can also be by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For oral administration, the oligomers are formulated into conventional oral administration forms such as capsules, tablets, and tonics. For topical administration, the oligomers of the invention are formulated into ointments, salves, gels, or creams as generally known in the art.

In addition to use in therapy, the oligomers of the invention may be used as diagnostic reagents to detect the presence or absence of the target DNA or RNA sequences to which they specifically bind. Such diagnostic tests are described in further detail below.

Likewise, the antisense constructs of the present invention, by antagonizing the normal biological activity of one of the *HDx* proteins, can be used in the manipulation of tissue, e.g. tissue differentiation or growth, both *in vivo* and *ex vivo*.

Furthermore, the anti-sense techniques (e.g. microinjection of antisense molecules, or transfection with plasmids whose transcripts are anti-sense with regard to an *HDx* mRNA or gene sequence) can be used to investigate role of *HDx* in



developmental events, as well as the normal cellular function of *HDx* in adult tissue. Such techniques can be utilized in cell culture, but can also be used in the creation of transgenic animals (described *infra*).

This invention also provides expression vectors containing a nucleic acid  
5 encoding an *HDx* polypeptide, operably linked to at least one transcriptional regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of the subject *HDx* proteins. Accordingly, the term transcriptional regulatory sequence includes  
10 promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences, sequences that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences  
15 encoding *HDx* polypeptides of this invention. Such useful expression control sequences, include, for example, a viral LTR, such as the LTR of the Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter  
20 regions of phage  $\lambda$ , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast  $\alpha$ -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It  
25 should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In one embodiment, the expression vector includes a  
30 recombinant gene encoding a peptide having an agonistic activity of a subject *HDx* polypeptide, or alternatively, encoding a peptide which is an antagonistic form of the *HDx* protein, such as a catalytically-inactive deacetylase. Such expression vectors can be used to transfect cells and thereby produce polypeptides, including fusion proteins, encoded by nucleic acids as described herein.

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Moreover, the gene constructs of the present invention can also be used as a part of a gene therapy protocol to deliver nucleic acids, e.g., encoding either an agonistic or antagonistic form of one of the subject *HDx* proteins or an antisense molecule described above. Thus, another aspect of the invention features expression vectors for *in vivo* or *in vitro* transfection and expression of an *HDx* polypeptide or antisense molecule in particular cell types so as to reconstitute the function of, or alternatively, abrogate the function of *HDx*-induced transcription in a tissue in which the naturally-occurring form of the protein is misexpressed; or to deliver a form of the protein which alters differentiation of tissue, or which inhibits neoplastic transformation.

Expression constructs of the subject *HDx* polypeptides, as well as antisense constructs, may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the recombinant gene to cells *in vivo*. Approaches include insertion of the subject gene in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or  $\text{CaPO}_4$  precipitation carried out *in vivo*. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically. Furthermore, it will be recognized that the particular gene construct provided for *in vivo* transduction of *HDx* expression are also useful for *in vitro* transduction of cells, such as for use in the *ex vivo* tissue culture systems described below.

A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA encoding the particular *HDx* polypeptide desired. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid. Retrovirus vectors, adenovirus vectors and adeno-associated virus vectors are exemplary recombinant gene delivery system for the transfer of exogenous genes *in vivo*,



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particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host.

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a subject *HDx* polypeptide in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the subject *HDx* polypeptide gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In clinical settings, the gene delivery systems for the therapeutic *HDx* gene can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) PNAS 91: 3054-3057). AN *HDx* gene, such as any one of the clones represented in the group consisting of SEQ ID NO:1-4, can be delivered in a gene therapy construct by electroporation using techniques described, for example, by Dev et al. ((1994) Cancer Treat Rev 20:105-115).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

Another aspect of the present invention concerns recombinant forms of the *HDx* proteins. Recombinant polypeptides preferred by the present invention, in addition to native *HDx* proteins, are at least 80% homologous, more preferably at least 85% homologous and most preferably at least 88% homologous with an amino acid sequence

represented by any of SEQ ID Nos: 5-8. Polypeptides which possess an activity of an *HDx* protein (i.e. either agonistic or antagonistic), and which are at least 90%, more preferably at least 95%, and most preferably at least about 98-99% homologous with a sequence selected from the group consisting of SEQ ID Nos: 5-8 are also within the scope of the invention. In other preferred embodiments, the *HDx* polypeptide includes both the  $\nu$  and  $\chi$  motifs, and preferably possess a histone deacetylase activity.

The term "recombinant *HDx* protein" refers to a polypeptide which is produced by recombinant DNA techniques, wherein generally, DNA encoding an *HDx* polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant *HDx* gene, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of a native *HDx* protein, or an amino acid sequence similar thereto which is generated by mutations including substitutions and deletions (including truncation) of a naturally occurring form of the protein.

The present invention further pertains to recombinant forms of the subject *HDx* polypeptides which are encoded by genes derived from a mammal (e.g. a human), and which have amino acid sequences evolutionarily related to the *HDx* proteins represented in SEQ ID Nos: 5-8. Such recombinant *HDx* polypeptides preferably are capable of functioning in one of either role of an agonist or antagonist of at least one biological activity of a wild-type ("authentic") *HDx* protein of the appended sequence listing. The term "evolutionarily related to", with respect to amino acid sequences of *HDx* proteins, refers to both polypeptides having amino acid sequences which have arisen naturally, and also to mutational variants of *HDx* polypeptides which are derived, for example, by combinatorial mutagenesis.

The present invention also provides methods of producing the subject *HDx* polypeptides. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the subject polypeptides can be cultured under appropriate conditions to allow expression of the peptide to occur. The cells may be harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The recombinant *HDx* polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immun affinity purification with antibodies specific for such peptide. In a preferred

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embodiment, the recombinant *HDx* polypeptide is a fusion protein containing a domain which facilitates its purification, such as GST fusion protein or poly(His) fusion protein.

This invention also pertains to a host cell transfected to express recombinant forms of the subject *HDx* polypeptides. The host cell may be any prokaryotic or eukaryotic cell. Thus, a nucleotide sequence derived from the cloning of *HDx* proteins, encoding all or a selected portion of a full-length protein, can be used to produce a recombinant form of an *HDx* polypeptide via microbial or eukaryotic cellular processes. Ligating the polynucleotide sequence into a gene construct, such as an expression vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect or mammalian) or prokaryotic (bacterial cells), are standard procedures used in producing other well-known proteins, e.g. MAP kinases, p53, WT1, PTP phosphatases, SRC, and the like. Similar procedures, or modifications thereof, can be employed to prepare recombinant *HDx* polypeptides by microbial means or tissue-culture technology in accord with the subject invention.

The recombinant *HDx* genes can be produced by ligating nucleic acid encoding an *HDx* protein, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vectors for production of recombinant forms of the subject *HDx* polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of an *HDx* polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach et al. (1983) in *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used. In an illustrative embodiment, an *HDx* polypeptide is produced recombinantly utilizing an expression vector generated by sub-cloning the coding sequence of one of the *HDx* genes represented in SEQ ID Nos:1-4.

The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic

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transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified  
5 with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and  
10 transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

In some instances, it may be desirable to express the recombinant *HDx*  
15 polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the  $\beta$ -gal containing pBlueBac III).

When it is desirable to express only a portion of an *HDx* protein, such as a form  
20 lacking a portion of the N-terminus, i.e. a truncation mutant which lacks the signal peptide, it may be necessary to add a start codon (ATG) to the oligonucleotide fragment containing the desired sequence to be expressed. It is well known in the art that a methionine at the N-terminal position can be enzymatically cleaved by the use of the enzyme methionine aminopeptidase (MAP). MAP has been cloned from *E. coli* (Ben-  
25 Bassat et al. (1987) J. Bacteriol. 169:751-757) and *Salmonella typhimurium* and its *in vitro* activity has been demonstrated on recombinant proteins (Miller et al. (1987) PNAS 84:2718-1722). Therefore, removal of an N-terminal methionine, if desired, can be achieved either *in vivo* by expressing *HDx*-derived polypeptides in a host which produces MAP (e.g., *E. coli* or CM89 or *S. cerevisiae*), or *in vitro* by use of purified MAP (e.g.,  
30 procedure of Miller et al., supra).

Alternatively, the coding sequences for the polypeptide can be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. This type of expression system can be useful under conditions where it is desirable to produce an immunogenic fragment of an *HDx* protein. For example, the VP6 capsid  
35 protein of rotavirus can be used as an immunologic carrier protein for portions of the

*HDx* polypeptide, either in the monomeric form or in the form of a viral particle. The nucleic acid sequences corresponding to the portion of a subject *HDx* protein to which antibodies are to be raised can be incorporated into a fusion gene construct which includes coding sequences for a late vaccinia virus structural protein to produce a set of recombinant viruses expressing fusion proteins comprising *HDx* epitopes as part of the virion. It has been demonstrated with the use of immunogenic fusion proteins utilizing the Hepatitis B surface antigen fusion proteins that recombinant Hepatitis B virions can be utilized in this role as well. Similarly, chimeric constructs coding for fusion proteins containing a portion of an *HDx* protein and the poliovirus capsid protein can be created to enhance immunogenicity of the set of polypeptide antigens (see, for example, EP Publication No: 0259149; and Evans et al. (1989) Nature 339:385; Huang et al. (1988) J. Virol. 62:3855; and Schlienger et al. (1992) J. Virol. 66:2).

The Multiple Antigen Peptide system for peptide-based immunization can also be utilized to generate an immunogen, wherein a desired portion of an *HDx* polypeptide is obtained directly from organo-chemical synthesis of the peptide onto an oligomeric branching lysine core (see, for example, Posnett et al. (1988) JBC 263:1719 and Nardelli et al. (1992) J. Immunol. 148:914). Antigenic determinants of *HDx* proteins can also be expressed and presented by bacterial cells.

In addition to utilizing fusion proteins to enhance immunogenicity, it is widely appreciated that fusion proteins can also facilitate the expression of proteins, and accordingly, can be used in the expression of the *HDx* polypeptides of the present invention. For example, *HDx* polypeptides can be generated as glutathione-S-transferase (GST-fusion) proteins. Such GST-fusion proteins can enable easy purification of the *HDx* polypeptide, as for example by the use of glutathione-derivatized matrices (see, for example, Current Protocols in Molecular Biology, eds. Ausubel et al. (N.Y.: John Wiley & Sons, 1991)).

In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence at the N-terminus of the desired portion of the recombinant protein, can allow purification of the expressed fusion protein by affinity chromatography using a Ni<sup>2+</sup> metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase to provide the purified protein (e.g., see Hochuli et al. (1987) J. Chromatography 411:177; and Janknecht et al. PNAS 88:8972).



Techniques for making fusion genes are known to those skilled in the art. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, Current Protocols in Molecular Biology, eds. Ausubel et al. John Wiley & Sons: 1992).

*HDx* polypeptides may also be chemically modified to create *HDx* derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of *HDx* proteins can be prepared by linking the chemical moieties to functional groups on amino acid sidechains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

The present invention also makes available isolated *HDx* polypeptides which are isolated from, or otherwise substantially free of other cellular proteins, especially other signal transduction factors and/or transcription factors which may normally be associated with the *HDx* polypeptide. The term "substantially free of other cellular proteins" (also referred to herein as "contaminating proteins") or "substantially pure or purified preparations" are defined as encompassing preparations of *HDx* polypeptides having less than 20% (by dry weight) contaminating protein, and preferably having less than 5% contaminating protein. Functional forms of the subject polypeptides can be prepared, for the first time, as purified preparations by using a cloned gene as described herein. By "purified", it is meant, when referring to a peptide or DNA or RNA sequence, that the indicated molecule is present in the substantial absence of other biological macromolecules, such as other proteins. The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein preferably has the same numerical limits as "purified" immediately above. "Isolated" and

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"purified" do not encompass either natural materials in their native state or natural materials that have been separated into components (e.g., in an acrylamide gel) but not obtained either as pure (e.g. lacking contaminating proteins, or chromatography reagents such as denaturing agents and polymers, e.g. acrylamide or agarose) substances or solutions. In preferred embodiments, purified *HDx* preparations will lack any contaminating proteins from the same animal from that *HDx* is normally produced, as can be accomplished by recombinant expression of, for example, a human *HDx* protein in a non-human cell.

As described above for recombinant polypeptides, isolated *HDx* polypeptides can include all or a portion of an amino acid sequences corresponding to an *HDx* polypeptide represented in any one of SEQ ID Nos: 5-8 or homologous sequences thereto. In preferred embodiments, the *HDx* polypeptide includes both the  $\nu$  and  $\chi$  motifs, and preferably possess a histone deacetylase activity.

Isolated peptidyl portions of *HDx* proteins can be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, an *HDx* polypeptide of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of a wild-type (e.g., "authentic") *HDx* protein.

The recombinant *HDx* polypeptides of the present invention also include homologs of the authentic *HDx* proteins, such as versions of those protein which are resistant to proteolytic cleavage, as for example, due to mutations which alter ubiquitination or other enzymatic targeting associated with the protein.

Modification of the structure of the subject *HDx* polypeptides can be for such purposes as enhancing therapeutic or prophylactic efficacy, stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*), or post-translational modifications (e.g., to alter phosphorylation pattern of protein). Such modified peptides, when designed to retain at least one activity of the naturally-occurring form of the protein, or to produce specific antagonists thereof, are considered functional equivalents of the *HDx*

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polypeptides described in more detail herein. Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition.

For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (i.e. isosteric and/or isoelectric mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur -containing = cysteine and methionine. (see, for example, Biochemistry, 2nd ed., Ed. by L. Stryer, WH Freeman and Co.: 1981). Whether a change in the amino acid sequence of a peptide results in a functional *HDx* homolog (e.g. functional in the sense that the resulting polypeptide mimics or antagonizes the wild-type form) can be readily determined by assessing the ability of the variant peptide to produce a response in cells in a fashion similar to the wild-type protein, or competitively inhibit such a response. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

This invention further contemplates a method for generating sets of combinatorial mutants of the subject *HDx* proteins as well as truncation mutants, and is especially useful for identifying potential variant sequences (e.g. homologs) that are functional in modulating histone deacetylation. The purpose of screening such combinatorial libraries is to generate, for example, novel *HDx* homologs which can act as either agonists or antagonist, or alternatively, possess novel activities all together. To illustrate, *HDx* homologs can be engineered by the present method to provide selective, constitutive activation of enzymatic activity. Thus, combinatorially-derived homologs can be generated to have an increased potency relative to a naturally occurring form of the protein.

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Likewise, *HDx* homologs can be generated by the present combinatorial approach to selectively inhibit (antagonize) histone deacetylation. For instance, mutagenesis can provide *HDx* homologs which are able to bind other regulatory proteins or cytoskeletal elements (or DNA) yet prevent acetylation of histones, e.g. the homologs can be  
 5 dominant negative mutants. In a preferred embodiment, a dominant negative mutant of an *HDx* protein is mutated at one or more residues of its catalytic site and/or specificity subsites.

In one aspect of this method, the amino acid sequences for a population of *HDx* homologs or other related proteins are aligned, preferably to promote the highest  
 10 homology possible. Such a population of variants can include, for example, *HDx* homologs from one or more species. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. In a preferred embodiment, the variegated library of *HDx* variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene  
 15 library. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential *HDx* sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display) containing the set of *HDx* sequences therein.

As illustrated in Figure 5B, to analyze the sequences of a population of variants,  
 20 the amino acid sequences of interest can be aligned relative to sequence homology. The presence or absence of amino acids from an aligned sequence of a particular variant is relative to a chosen consensus length of a reference sequence, which can be real or artificial. For instance, Figure 5B includes the alignment of the  $\nu$  and  $\chi$ -motifs for several of the *HDx* gene products. Analysis of the alignment of these sequences from the *HDx*  
 25 clones can give rise to the generation of a degenerate library of polypeptides comprising potential *HDx* sequences. In an exemplary embodiment, a library of variants based on the *HD1* sequence, but degenerate across each of the  $\nu$  and  $\chi$ -motifs can be provided. On such library can be represented by the general formula A-( $\nu$  motif)-B-( $\chi$  motif)-C, wherein the  $\nu$  motif is an amino acid sequence represented in the general formula

30 DIAX<sub>1</sub>NWAGGLHHAKKX<sub>2</sub>EASGFCYVNDIVX<sub>3</sub>X<sub>4</sub>ILELLKYHX<sub>5</sub>RVLYIDIDIHHGDGX<sub>6</sub>EEAFYX  
 7TD-RVMTVSF

the  $\chi$  motif is an amino acid sequence represented in the general formula

CVEX<sub>8</sub>VKX<sub>9</sub>FNX<sub>10</sub>PLLX<sub>11</sub>LGGGGYTX<sub>12</sub>RNVARCWTYET

A corresponds to Met1-Thr129 of SEQ ID No. 5, B corresponds to His199-Lys283 of SEQ ID No. 5, and C corresponds to Ala317-Ala482 of SEQ ID No. 5, wherein X<sub>1</sub> represents Ile or Val; X<sub>2</sub> represents Phe or Ser; X<sub>3</sub> represents Phe or Leu; X<sub>4</sub> represents Gly or Ala; X<sub>5</sub> represents Pro or Gln; X<sub>6</sub> represents Gln or Glu; X<sub>7</sub> represents Leu or Thr; X<sub>8</sub> represents Val or Tyr; X<sub>9</sub> represents Thr or Ser; X<sub>10</sub> represents Leu or Ile; X<sub>11</sub> represents Met or Val; and X<sub>12</sub> represents Ile or Val. To further expand the combinatorial set, other conservative mutations relative to those appearing in the human sequences can be provided. For example, in a more expansive library, X<sub>1</sub> represents Gly, Ala, Val, Ile or Leu; X<sub>2</sub> represents Phe, Tyr, Thr or Ser; X<sub>3</sub> represents Phe, Tyr, Gly, Ala, Val, Ile or Leu; X<sub>4</sub> represents Gly, Ala, Val, Ile or Leu; X<sub>5</sub> represents Pro, Asn or Gln; X<sub>6</sub> represents Asn, Gln, Asp or Glu; X<sub>7</sub> represents Gly, Ala, Val, Ile, Leu, Ser or Thr; X<sub>8</sub> represents Gly, Ala, Val, Ile, Leu, Phe or Tyr; X<sub>9</sub> represents Thr, Cys, or Ser; X<sub>10</sub> represents Gly, Ala, Val, Ile or Leu; X<sub>11</sub> represents Met, Cys, Gly, Ala, Val, Ile, Leu, Ser or Thr; and X<sub>12</sub> represents Gly, Ala, Val, Ile or Leu. In still another library, each degenerate position can be any one of the naturally occurring amino acids. Likewise, the  $\nu$  and  $\chi$ -motifs can correspond to the degenerate sequences designated by SEQ ID Nos. 12 and 14, respectively.

There are many ways by which such libraries of potential *HDx* homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential *HDx* sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al. (1981) Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) Science 249:386-390; Roberts et al. (1992) PNAS 89:2429-2433; Devlin et al. (1990) Science 249: 404-406; Cwirla et al. (1990) PNAS 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

Likewise, a library of coding sequence fragments can be provided for an *HDx* clone in order to generate a variegated population of *HDx* fragments for screening and subsequent selection of bioactive fragments. A variety of techniques are known in the art for generating such libraries, including chemical synthesis. In one embodiment, a library



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of coding sequence fragments can be generated by (i) treating a double stranded PCR fragment of an *HDx* coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule; (ii) denaturing the double stranded DNA; (iii) renaturing the DNA to form double stranded DNA which can include sense/antisense  
5 pairs from different nicked products; (iv) removing single stranded portions from reformed duplexes by treatment with S1 nuclease; and (v) ligating the resulting fragment library into an expression vector. By this exemplary method, an expression library can be derived which codes for N-terminal, C-terminal and internal fragments of various sizes.

A wide range of techniques are known in the art for screening gene products of  
10 combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of *HDx* homologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression  
15 vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected.

In an exemplary embodiment, the library of *HDx* variants is expressed as a fusion  
20 protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at very high concentrations, a large number of phage can be screened at one time. Second, since each infectious phage displays the combinatorial gene product on its  
25 surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E. coli* filamentous phages M13, fd., and f1 are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al. PCT  
30 publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) *J. Biol. Chem.* 267:16007-16010; Griffiths et al. (1993) *EMBO J* 12:725-734; Clackson et al. (1991) *Nature* 352:624-628; and Barbas et al. (1992) *PNAS* 89:4457-4461).

For example, the recombinant phage antibody system (RPAS, Pharmacia Catalog  
35 number 27-9400-01) can be easily modified for use in expressing and screening *HDx*

combinatorial libraries by panning on glutathione immobilized histones/GST fusion proteins or RbAp48/GST fusion protein to enrich for *HDx* homologs which retain an ability to bind a substrate or regulatory protein. Each of these *HDx* homologs can subsequently be screened for further biological activities in order to differentiate agonists and antagonists. For example, histone-binding homologs isolated from the combinatorial library can be tested for their enzymatic activity directly, or for their effect on cellular proliferation relative to the wild-type form of the protein.

The invention also provides for reduction of the *HDx* or RbAp48 or histones proteins to generate mimetics, e.g. peptide or non-peptide agents, which are able to disrupt a biological activity of an *HDx* polypeptide of the present invention, e.g. as catalytic inhibitor or an inhibitor of protein-protein interactions. Thus, such mutagenic techniques as described above are also useful to map the determinants of the *HDx* proteins which participate in protein-protein or protein-DNA interactions involved in, for example, interaction of the subject *HDx* polypeptide with histones, RbAp48 or cytoskeletal elements. To illustrate, the critical residues of a subject *HDx* polypeptide which are involved in molecular recognition of histones can be determined and used to generate *HDx*-derived peptidomimetics which competitively inhibit binding of the authentic *HDx* protein with that moiety. Likewise, residues of a histone or of RbAp48 involved in binding to *HDx* proteins can be identified, and peptides or peptidomimetics based on such residues can also be used as competitive inhibitors of the interaction of an *HDx* protein with either of those proteins. By employing, for example, scanning mutagenesis to map the amino acid residues of a protein which is involved in binding other proteins, peptidomimetic compounds can be generated which mimic those residues which facilitate the interaction. Such mimetics may then be used to interfere with the normal function of an *HDx* protein. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985),  $\beta$ -turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), and  $\beta$ -aminoalcohols

(Gordon et al. (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71).

Another aspect of the invention pertains to an antibody specifically reactive with an *HDx* protein. For example, by using immunogens derived from an *HDx* protein, e.g. based on the cDNA sequences, anti-protein/anti-peptide antisera or monoclonal antibodies can be made by standard protocols (See, for example, *Antibodies: A Laboratory Manual* ed. by Harlow and Lane (Cold Spring Harbor Press: 1988)). A mammal, such as a mouse, a hamster or rabbit can be immunized with an immunogenic form of the peptide (e.g., an *HDx* polypeptide or an antigenic fragment which is capable of eliciting an antibody response). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. An immunogenic portion of an *HDx* protein can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies. In a preferred embodiment, the subject antibodies are immunospecific for antigenic determinants of an *HDx* protein of a organism, such as a mammal, e.g. antigenic determinants of a protein represented by one of SEQ ID Nos: 5-8 or closely related homologs (e.g. at least 85% homologous, preferably at least 90% homologous, and more preferably at least 95% homologous). In yet a further preferred embodiment of the present invention, in order to provide, for example, antibodies which are immuno-selective for discrete *HDx* homologs, e.g. *HD1*, the anti-*HDx* polypeptide antibodies do not substantially cross react (i.e. does not react specifically) with a protein which is, for example, less than 85%, 90% or 95% homologous with the selected *HDx*. By "not substantially cross react", it is meant that the antibody has a binding affinity for a non-homologous protein which is at least one order of magnitude, more preferably at least 2 orders of magnitude, and even more preferably at least 3 orders of magnitude less than the binding affinity of the antibody for the intended target *HDx*.

Following immunization of an animal with an antigenic preparation of an *HDx* polypeptide, anti-*HDx* antisera can be obtained and, if desired, polyclonal anti-*HDx* antibodies isolated from the serum. To produce monoclonal antibodies, antibody-producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique (originally developed by Kohler and Milstein, (1975)

Nature, 256: 495-497), the human B cell hybridoma technique (Kozbar et al., (1983) Immunology Today, 4: 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., (1985) Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. pp. 77-96). Hybridoma cells can be screened immunochemically for  
5 production of antibodies specifically reactive with an *HDx* polypeptide of the present invention and monoclonal antibodies isolated from a culture comprising such hybridoma cells.

The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with one of the subject *HDx* polypeptides. Antibodies can be  
10 fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example,  $F(ab)_2$  fragments can be generated by treating antibody with pepsin. The resulting  $F(ab)_2$  fragment can be treated to reduce disulfide bridges to produce Fab fragments. The antibody of the present invention is further intended to include bispecific and chimeric molecules having  
15 affinity for an *HDx* protein conferred by at least one CDR region of the antibody.

Both monoclonal and polyclonal antibodies (Ab) directed against authentic *HDx* polypeptides, or *HDx* variants, and antibody fragments such as Fab,  $F(ab)_2$ , Fv and scFv can be used to block the action of one or more *HDx* proteins and allow the study of the role of these proteins in, for example, differentiation of tissue. Experiments of this nature  
20 can aid in deciphering the role of *HDx* proteins that may be involved in control of proliferation versus differentiation, e.g., in patterning and tissue formation.

Antibodies which specifically bind *HDx* epitopes can also be used in immunohistochemical staining of tissue samples in order to evaluate the abundance and pattern of expression of each of the subject *HDx* polypeptides. Anti-*HDx* antibodies can  
25 be used diagnostically in immuno-precipitation and immuno-blotting to detect and evaluate *HDx* protein levels in tissue as part of a clinical testing procedure. For instance, such measurements can be useful in predictive valuations of the onset or progression of proliferative or differentiative disorders. Likewise, the ability to monitor *HDx* protein levels in an individual can allow determination of the efficacy of a given treatment  
30 regimen for an individual afflicted with such a disorder. The level of *HDx* polypeptides may be measured from cells in bodily fluid, such as in samples of cerebral spinal fluid or amniotic fluid, or can be measured in tissue, such as produced by biopsy. Diagnostic assays using anti-*HDx* antibodies can include, for example, immunoassays designed to aid in early diagnosis of a disorder, particularly ones which are manifest at birth. Diagnostic

assays using anti-*HDx* polypeptide antibodies can also include immunoassays designed to aid in early diagnosis and phenotyping neoplastic or hyperplastic disorders.

Another application of anti-*HDx* antibodies of the present invention is in the immunological screening of cDNA libraries constructed in expression vectors such as  $\lambda$ gt11,  $\lambda$ gt18-23,  $\lambda$ ZAP, and  $\lambda$ ORF8. Messenger libraries of this type, having coding sequences inserted in the correct reading frame and orientation, can produce fusion proteins. For instance,  $\lambda$ gt11 will produce fusion proteins whose amino termini consist of  $\beta$ -galactosidase amino acid sequences and whose carboxy termini consist of a foreign polypeptide. Antigenic epitopes of an *HDx* protein, e.g. other orthologs of a particular *HDx* protein or other paralogs from the same species, can then be detected with antibodies, as, for example, reacting nitrocellulose filters lifted from infected plates with anti-*HDx* antibodies. Positive phage detected by this assay can then be isolated from the infected plate. Thus, the presence of *HDx* homologs can be detected and cloned from other animals, as can alternate isoforms (including splicing variants) from humans.

Moreover, the nucleotide sequences determined from the cloning of *HDx* genes from organisms will further allow for the generation of probes and primers designed for use in identifying and/or cloning *HDx* homologs in other cell types, e.g. from other tissues, as well as *HDx* homologs from other organisms. For instance, the present invention also provides a probe/primer comprising a substantially purified oligonucleotide, which oligonucleotide comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least 10 consecutive nucleotides of sense or anti-sense sequence selected from the group consisting of SEQ ID Nos: 1-4 or naturally occurring mutants thereof. For instance, primers based on the nucleic acid represented in SEQ ID Nos: 1-4 can be used in PCR reactions to clone *HDx* homologs. Likewise, probes based on the subject *HDx* sequences can be used to detect transcripts or genomic sequences encoding the same or homologous proteins. In preferred embodiments, the probe further comprises a label group attached thereto and able to be detected, e.g. the label group is selected from amongst radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors.

Such probes can also be used as a part of a diagnostic test kit for identifying cells or tissue which misexpress an *HDx* protein, such as by measuring a level of an *HDx*-encoding nucleic acid in a sample of cells from a patient; e.g. detecting *HDx* mRNA levels or determining whether a genomic *HDx* gene has been mutated or deleted.



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To illustrate, nucleotide probes can be generated from the subject *HDx* genes which facilitate histological screening of intact tissue and tissue samples for the presence (or absence) of *HDx*-encoding transcripts. Similar to the diagnostic uses of anti-*HDx* antibodies, the use of probes directed to *HDx* messages, or to genomic *HDx* sequences, can be used for both predictive and therapeutic evaluation of allelic mutations which might be manifest in, for example, neoplastic or hyperplastic disorders (e.g. unwanted cell growth) or abnormal differentiation of tissue. Used in conjunction with immunoassays as described above, the oligonucleotide probes can help facilitate the determination of the molecular basis for a developmental disorder which may involve some abnormality associated with expression (or lack thereof) of an *HDx* protein. For instance, variation in polypeptide synthesis can be differentiated from a mutation in a coding sequence.

Accordingly, the present method provides a method for determining if a subject is at risk for a disorder characterized by aberrant cell proliferation and/or differentiation. In preferred embodiments, method can be generally characterized as comprising detecting, in a sample of cells from the subject, the presence or absence of a genetic lesion characterized by at least one of (i) an alteration affecting the integrity of a gene encoding an *HDx*-protein, or (ii) the mis-expression of the *HDx* gene. To illustrate, such genetic lesions can be detected by ascertaining the existence of at least one of (i) a deletion of one or more nucleotides from an *HDx* gene, (ii) an addition of one or more nucleotides to an *HDx* gene, (iii) a substitution of one or more nucleotides of an *HDx* gene, (iv) a gross chromosomal rearrangement of an *HDx* gene, (v) a gross alteration in the level of a messenger RNA transcript of an *HDx* gene, (vi) aberrant modification of an *HDx* gene, such as of the methylation pattern of the genomic DNA, (vii) the presence of a non-wild type splicing pattern of a messenger RNA transcript of an *HDx* gene, (viii) a non-wild type level of an *HDx*-protein, and (ix) inappropriate post-translational modification of an *HDx*-protein. As set out below, the present invention provides a large number of assay techniques for detecting lesions in an *HDx* gene, and importantly, provides the ability to discern between different molecular causes underlying *HDx*-dependent aberrant cell growth, proliferation and/or differentiation.

In an exemplary embodiment, there is provided a nucleic acid composition comprising a (purified) oligonucleotide probe including a region of nucleotide sequence which is capable of hybridizing to a sense or antisense sequence of an *HDx* gene, such as represented by any of SEQ ID Nos: 1-4, or naturally occurring mutants thereof, or 5' or 3' flanking sequences or intronic sequences naturally associated with the subject *HDx* genes or naturally occurring mutants thereof. The nucleic acid of a cell is rendered

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accessible for hybridization, the probe is exposed to nucleic acid of the sample, and the hybridization of the probe to the sample nucleic acid is detected. Such techniques can be used to detect lesions at either the genomic or mRNA level, including deletions, substitutions, etc., as well as to determine mRNA transcript levels.

5 In certain embodiments, detection of the lesion comprises utilizing the probe/primer in a polymerase chain reaction (PCR) (see, e.g. U.S. Patent Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al. (1988) Science 241:1077-1080; and Nakazawa et al. (1944) PNAS 91:360-364), the later of which can be particularly useful  
10 for detecting point mutations in the *HDx* gene. In a merely illustrative embodiment, the method includes the steps of (i) collecting a sample of cells from a patient, (ii) isolating nucleic acid (e.g., genomic, mRNA or both) from the cells of the sample, (iii) contacting the nucleic acid sample with one or more primers which specifically hybridize to an *HDx* gene under conditions such that hybridization and amplification of the *HDx* gene (if  
15 present) occurs, and (iv) detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample.

In still another embodiment, the level of an *HDx*-protein can be detected by immunoassay. For instance, the cells of a biopsy sample can be lysed, and the level of an  
20 *HDx*-protein present in the cell can be quantitated by standard immunoassay techniques. In yet another exemplary embodiment, aberrant methylation patterns of an *HDx* gene can be detected by digesting genomic DNA from a patient sample with one or more restriction endonucleases that are sensitive to methylation and for which recognition sites exist in the *HDx* gene (including in the flanking and intronic sequences). See, for  
25 example, Buiting et al. (1994) Human Mol Genet 3:893-895. Digested DNA is separated by gel electrophoresis, and hybridized with probes derived from, for example, genomic or cDNA sequences. The methylation status of the *HDx* gene can be determined by comparison of the restriction pattern generated from the sample DNA with that for a standard of known methylation.

30 In yet another aspect of the invention, the subject *HDx* polypeptides can be used to generate a "two hybrid" assay or an "interaction trap" assay (see, for example, U.S. Patent No. 5,283,317; Zervos et al. (1993) Cell 72:223-232; Madura et al. (1993) J Biol Chem 268:12046-12054; Bartel et al. (1993) Biotechniques 14:920-924; Iwabuchi et al. (1993) Oncogene 8:1693-1696; and Brent WO94/10300), for isolating coding sequences  
35 for other cellular proteins which bind *HDxs* ("*HDx*-binding proteins" or "*HDx*-bp").

Such *HDx*-binding proteins would likely be involved in the regulation of *HDx*, e.g., as regulatory subunits or transducers, or be substrates which are regulated by an *HDx*.

Briefly, the interaction trap relies on reconstituting *in vivo* a functional transcriptional activator protein from two separate fusion proteins. In particular, the method makes use of chimeric genes which express hybrid proteins. To illustrate, a first hybrid gene comprises the coding sequence for a DNA-binding domain of a transcriptional activator fused in frame to the coding sequence for an *HDx* polypeptide. The second hybrid protein encodes a transcriptional activation domain fused in frame to a sample gene from a cDNA library. If the bait and sample hybrid proteins are able to interact, e.g., form an *HDx*-dependent complex, they bring into close proximity the two domains of the transcriptional activator. This proximity is sufficient to cause transcription of a reporter gene which is operably linked to a transcriptional regulatory site responsive to the transcriptional activator, and expression of the reporter gene can be detected and used to score for the interaction of the *HDx* and sample proteins.

Furthermore, by making available purified and recombinant *HDx* polypeptides, the present invention facilitates the development of assays which can be used to screen for drugs, including *HDx* homologs, which are either agonists or antagonists of the normal cellular function of the subject *HDx* polypeptides, or of their role in the pathogenesis of cellular differentiation and/or proliferation and disorders related thereto. Moreover, because we have also identified *HDx*-related proteins, such as the yeast RPD3 proteins, as histone deacetylases, the present invention further provides drug screening assays for detecting agents which modulate the bioactivity of *HDx*-related proteins. Such agents, when directed to, for example, fungal *HDx*-related proteins, can be used in the treatment of various infections. In a general sense, the assay evaluates the ability of a compound to modulate binding between an *HDx* polypeptide and a molecule, be it protein or DNA, that interacts with the *HDx* polypeptide. It will be apparent from the following description of exemplary assays that, in place of a human (or other mammalian) *HDx* protein, the assay can be derived with an *HDx*-related protein such as RPD3. Likewise, in place of human RbAp48 or Sin3A, other *HDx*-binding proteins can be used, e.g., other human proteins. Exemplary compounds which can be screened include peptides, nucleic acids, carbohydrates, small organic molecules, and natural product extract libraries, such as isolated from animals, plants, fungus and/or microbes.

It is contemplated that any of the novel interactions described herein could be exploited in a drug screening assay. For example, in one embodiment, the interaction between an *HDx* protein and RbAp48 can be detected in the presence and the absence of

a test compound. In another embodiment, the ability of a compound to modulate the binding of an *HDx* protein, or *HDx*-related protein such as the yeast RPD3, with histones can be assessed. The identification of a test compound which influences, for example, HD1 catalyzed deacetylation of histones would be useful in the modulation of HD1 activity in mammalian cells, while the identification of a test compound which selectively inhibits the yeast RPD3 deacetylase activity would be useful as an antifungal agent. In other embodiments the effect of a test compound on the binding of an *HDx* protein to other molecules, such as cytoskeletal components, or other proteins identified by the *HDx*-dependent ITS set out above, could be tested. A variety of assay formats will suffice and, in light of the present inventions, will be comprehended by a skilled artisan.

In a preferred embodiment, assays which employ the subject mammalian *HDx* proteins can be used to identify compounds that have therapeutic indexes more favorable than sodium butyrate, trapoxin, trichostatin or the like. For instance, trapoxin-like drugs can be identified by the present invention which have enhanced tissue-type or cell-type specificity relative to trapoxin. To illustrate, the subject assays can be used to generate compounds which preferentially inhibit IL-2 mediated proliferation/activation of lymphocytes, or inhibit proliferation of certain tumor cells, without substantially interfering with other tissues, e.g. hepatocytes. Likewise, similar assays can be used to identify drugs which inhibit proliferation of yeast cells or other lower eukaryotes, but which have a substantially reduced effect on mammalian cells, thereby improving therapeutic index of the drug as an anti-mycotic agent.

In one embodiment, the identification of such compounds is made possible by the use of differential screening assays which detect and compare drug-mediated inhibition of deacetylase activity between two or more different *HDx*-like enzymes, or compare drug-mediated inhibition of formation of complexes involving two or more different types of *HDx*-like proteins. To illustrate, the assay can be designed for side-by-side comparison of the effect of a test compound on the deacetylase activity or protein interactions of tissue-type specific *HDx* proteins. Given the apparent diversity of *HDx* proteins, it is probable that different functional *HDx* activities, or *HDx* complexes exist and, in certain instances, are localized to particular tissue or cell types. Thus, test compounds can be screened for agents able to inhibit the tissue-specific formation of only a subset of the possible repertoire of *HDx*/regulatory protein complexes, or which preferentially inhibit certain *HDx* enzymes. In an exemplary embodiment, an interaction trap assay can be derived using two or more different human *HDx* "bait" proteins, while the "fish" protein is constant in each, e.g. a human RbAp48 construct. Running the interaction trap side-



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by-side permits the detection of agents which have a greater effect (e.g. statistically significant) on the formation of one of the *HDx*/RbAp48 complexes than on the formation of the other *HDx* complexes.

In similar fashion, differential screening assays can be used to exploit the difference in protein interactions and/or catalytic mechanism of mammalian *HDx* proteins and yeast *RPD3* proteins in order to identify agents which display a statistically significant increase in specificity for inhibiting the yeast enzyme relative to the mammalian enzyme. Thus, lead compounds which act specifically on pathogens, such as fungus involved in mycotic infections, can be developed. By way of illustration, the present assays can be used to screen for agents which may ultimately be useful for inhibiting at least one fungus implicated in such mycosis as candidiasis, aspergillosis, mucormycosis, blastomycosis, geotrichosis, cryptococcosis, chromoblastomycosis, coccidioidomycosis, conidiosporosis, histoplasmosis, maduromycosis, rhinosporidiosis, nocardiosis, para-actinomycosis, penicilliosis, monoliasis, or sporotrichosis. For example, if the mycotic infection to which treatment is desired is candidiasis, the present assay can comprise comparing the relative effectiveness of a test compound on inhibiting the deacetylase activity of a mammalian *HDx* protein with its effectiveness towards inhibiting the deacetylase activity of an *RPD3* homolog cloned from yeast selected from the group consisting of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quilliermondii*, or *Candida rugosa*. Likewise, the present assay can be used to identify anti-fungal agents which may have therapeutic value in the treatment of aspergillosis by selectively targeting *RPD3* homologs cloned from yeast such as *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, or *Aspergillus terreus*. Where the mycotic infection is mucormycosis, the *RPD3* deacetylase can be derived from yeast such as *Rhizopus arrhizus*, *Rhizopus oryzae*, *Absidia corymbifera*, *Absidia ramosa*, or *Mucor pusillus*. Sources of other *RPD3* activities for comparison with a mammalian *HDx* activity includes the pathogen *Pneumocystis carinii*.

In addition to such *HDx* therapeutic uses, anti-fungal agents developed with such differential screening assays can be used, for example, as preservatives in foodstuff, feed supplement for promoting weight gain in livestock, or in disinfectant formulations for treatment of non-living matter, e.g., for decontaminating hospital equipment and rooms.

In similar fashion, side by side comparison of inhibition of a mammalian *HDx* proteins and an insect *HDx*-related proteins, will permit selection of *HDx* inhibitors which discriminate between the human/mammalian and insect enzymes. Accordingly, the



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present invention expressly contemplates the use and formulations of the subject *HDx* therapeutics in insecticides, such as for use in management of insects like the fruit fly.

5 In yet another embodiment, certain of the subject *HDx* inhibitors can be selected on the basis of inhibitory specificity for plant *HDx*-related activities relative to the mammalian enzyme. For example, a plant *HDx*-related protein can be disposed in a differential screen with one or more of the human enzymes to select those compounds of greatest selectivity for inhibiting the plant enzyme. Thus, the present invention specifically contemplates formulations of the subject *HDx* inhibitors for agricultural applications, such as in the form of a defoliant or the like.

10 In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with upstream or downstream elements. Accordingly, in 20 an exemplary screening assay of the present invention, a reaction mixture is generated to include an *HDx* polypeptide, compound(s) of interest, and a "target polypeptide", e.g., a protein, which interacts with the *HDx* polypeptide, whether as a substrate or by some other protein-protein interaction. Exemplary target polypeptides include histones, RbAp48 polypeptides, Sin3 polypeptides, and/or combinations thereof or with other 25 transcriptional regulatory proteins (such as myc, max, etc, see Example 3)). Detection and quantification of complexes containing the *HDx* protein provide a means for determining a compound's efficacy at inhibiting (or potentiating) complex formation between the *HDx* and the target polypeptide. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various 30 concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, isolated and purified *HDx* polypeptide is added to a composition containing the target polypeptide and the formation of a complex is quantitated in the absence of the test compound.

35 Complex formation between the *HDx* polypeptide and the target polypeptide may be detected by a variety of techniques. Modulation of the formation of complexes can be

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quantitated using, for example, detectably labeled proteins such as radiolabeled, fluorescently labeled, or enzymatically labeled *HDx* polypeptides, by immunoassay, by chromatographic detection, or by detecting the intrinsic activity of the acetylase.

Typically, it will be desirable to immobilize either *HDx* or the target polypeptide to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of *HDx* to the target polypeptide, in the presence and absence of a candidate agent, can be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/*HDx* (GST/*HDx*) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the cell lysates, e.g. an <sup>35</sup>S-labeled, and the test compound, and the mixture incubated under conditions conducive to complex formation, e.g. at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound label, and the matrix immobilized and radiolabel determined directly (e.g. beads placed in scintillant), or in the supernatant after the complexes are subsequently dissociated. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of *HDx*-binding protein found in the bead fraction quantitated from the gel using standard electrophoretic techniques such as described in the appended examples.

Other techniques for immobilizing proteins on matrices are also available for use in the subject assay. For instance, either *HDx* or target polypeptide can be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated *HDx* molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with *HDx*, but which do not interfere with the interaction between the *HDx* and target polypeptide, can be derivatized to the wells of the plate, and *HDx* trapped in the wells by antibody conjugation. As above, preparations of an target polypeptide and a test compound are incubated in the *HDx*-presenting wells of the plate, and the amount of complex trapped in the well can be quantitated. Exemplary methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the target

polypeptide, or which are reactive with *HDx* protein and compete with the target polypeptide; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the target polypeptide, either intrinsic or extrinsic activity. In the instance of the latter, the enzyme can be chemically conjugated or provided as a fusion protein with the target polypeptide. To illustrate, the target polypeptide can be chemically cross-linked or genetically fused with horseradish peroxidase, and the amount of polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme, e.g. 3,3'-diamino-benzadine tetrahydrochloride or 4-chloro-1-naphthol. Likewise, a fusion protein comprising the polypeptide and glutathione-S-transferase can be provided, and complex formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig et al (1974) J Biol Chem 249:7130).

For processes which rely on immunodetection for quantitating one of the proteins trapped in the complex, antibodies against the protein, such as anti-*HDx* antibodies, can be used. Alternatively, the protein to be detected in the complex can be "epitope tagged" in the form of a fusion protein which includes, in addition to the *HDx* sequence, a second polypeptide for which antibodies are readily available (e.g. from commercial sources). For instance, the GST fusion proteins described above can also be used for quantification of binding using antibodies against the GST moiety. Other useful epitope tags include myc-epitopes (e.g., see Ellison et al. (1991) J Biol Chem 266:21150-21157) which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein A system (Pharmacia, NJ).

In another embodiment of a drug screening, a two hybrid assay can be generated with an *HDx* and *HDx*-binding protein. Drug dependent inhibition or potentiation of the interaction can be scored.

Where the *HDx* proteins themselves, or in complexes with other proteins, are capable of binding DNA and modifying transcription of a gene, a transcriptional based assay using, for example, an transcriptional regulatory sequences responsive to *HDx* complexes operably linked to a detectable marker gene. For illustration, see Example 3.

To test the effect of a histone deacetylase inhibitor on MadN35GALVP16 and Mad(Pro)N35GALVPI6 mediated repression, we treated a duplicate set of transfections with 10 nM trapoxin for eight hours prior to harvest. In the representative experiment shown, 10 nM trapoxin treatment derepressed the activity of MadN35GALVP16 nine-fold while it had little effect on the activity of Mad(Pro)N35GALVP16, suggesting that the histone deacetylation plays a direct role in mSin3A transcriptional repression (Figure

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13B). In addition, there was typically less than a two-fold effect of trapoxin on the activity of the reporter gene in cells transfected with the expression vector alone or in cells transfected with GALVP16 (data not shown). Following trapoxin treatment, the repression observed for MadN35GALVP16 was still seven times greater than that of  
5 Mad(Pro)N35GALVP16, suggesting that the residual deacetylase activity following trapoxin treatment (Figure 13B) continues to drive mSin3A-mediated repression; however, we can not rule out that mSin3A is capable of repression by mechanisms independent of histone deacetylation.

10 Furthermore, each of the assay systems set out above can be generated in a "differential" format as set forth above. That is, the assay format can provide information regarding specificity as well as potency. For instance, side-by-side comparison of a test compound's effect on different HDxs can provide information on selectivity, and permit the identification of compounds which selectively modulate the bioactivity of only a  
15 subset of the HDx family.

Furthermore, inhibitors of the enzymatic activity of each of the subject HDx proteins can be identified using assays derived from measuring the ability of an agent to inhibit catalytic conversion of a substrate by the subject proteins. For example, the ability of the subject HDx proteins to deacetylate a histone substrate, such as histone H4 (see  
20 examples), in the presence and absence of a candidate inhibitor, can be determined using standard enzymatic assays.

A number of methods have been employed in the art for assaying histone deacetylase activity, and can be incorporated in the drug screening assays of the present invention. In preferred embodiments, the assay will employ a labeled acetyl group linked  
25 to appropriate histone lysine residues as substrates. In other embodiments, a histone substrate peptide can be labeled with a group whose signal is dependent on the simultaneous presence or absence of an acetyl group, e.g., the label can be a fluorogenic group whose fluorescence is modulated (either quenched or potentiated) by the presence of the acetyl moiety. Using standard enzymatic analysis, the ability of a test agent to  
30 cause a statistically significant change in substrate conversion by a histone deacetylase can be measured, and as desirable, inhibition constants, e.g.,  $K_i$  values, can be calculated. The histone substrate can be provided as a purified or semi-purified polypeptide or as part of a cell lysate. Likewise, the histone deacetylase can be provided to the reaction mixture as a purified or semi-purified polypeptide or as a cell lysate. Accordingly, the

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reaction mixtures of the subject method can range from reconstituted protein mixtures derived with purified preparations of histones and deacetylases, to mixtures of cell lysates, e.g., by admixing baculovirus lysates containing recombinant histones and deacetylases.

- 5 In an exemplary embodiment, the histone substrate for the subject assay is provided by isolation of radiolabeled histones from metabolically labelled cells. To illustrate, as described by Hay et al. (1983) *J Biol Chem* 258:3726-3734, HeLa cells can be labelled in culture by addition of [ $^3\text{H}$ ]acetate (New England Nuclear) to the culture media. The addition of butyrate, trapoxin or the like can be used to increase the
- 10 abundance of acetylated histones in the cells. Radiolabelled histones can be isolated from the cells by extraction with  $\text{H}_2\text{SO}_4$  (Marushige et al. (1966) *J Mol Biol* 15:160-174). Briefly, cells are homogenized in buffer, centrifuged to isolate a nuclear pellet, the subsequently homogenized nuclear pellet centrifuged through sucrose, and the resulting chromatin pellet extracted by addition of  $\text{H}_2\text{SO}_4$  to yield [ $^3\text{H}$ ]acetyl-labelled histones. In
- 15 an alternate embodiment, nucleosome preparations containing [ $^3\text{H}$ ]acetyl-labelled histones can be isolated from the labelled cells. As described in the art, nucleosomes can be isolated from cell preparations by sucrose gradient centrifugation (Hay et al. (1983) *J Biol Chem* 258:3726-3734; and Noll (1967) *Nature* 215:360-363), and polynucleosomes can be prepared by NaCl precipitation from micrococcal nuclease digested cells (Hay et
- 20 al., *supra*). Similar procedures for isolating labelled histones from other cells types, including yeast, have been described. See, for example, Alonso et al. (1986) *Biochem Biophys Acta* 866:161-169; and Kreiger et al. (1974) *J Biol Chem* 249:332-334. In yet other embodiments, the histone is generated by recombinant gene expression, and includes an exogenous tag (e.g., an HA epitope, a poly(his) sequence or the like) which
- 25 facilitates in purification from cell extracts. In still other embodiments, whole nuclei can be isolated from metabolically labelled cells by micrococcal nuclease digestion (Hay et al., *supra*)

- In still another embodiment, the deacetylase substrate can be provided as an acetylated peptide including a sequence corresponding to the sequence about the specific
- 30 lysyl residues acetylated on histone, e.g., a peptidyl portions of the core histones H2A, H2B, H3 or H4. Such fragments can be produced by cleavage of acetylated histones derived from metabolically labelled cells, e.g., such as by treatment with proteolytic enzymes or cyanogen bromide (Kreiger et al., *supra*). In other embodiments, the acetylated peptide can be provided by standard solid phase synthesis using acetylated
- 35 lysine residues (Kreiger et al., *supra*).



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Continuing with the illustrative use of [ $^3\text{H}$ ]acetyl-labelled histones, the activity of a histone deacetylase in the subject assays is detected by measuring release of [ $^3\text{H}$ ]acetate by standard scintillant techniques. In a merely illustrative example, a reaction mixture is provided which comprises a recombinant *HDx* protein suspended in buffer, along with a sample of [ $^3\text{H}$ ]acetyl-labelled histones and (optionally) a test compound. The reaction mixture is maintained at a desired temperature and pH, such as 22°C at pH7.8, for several hours, and the reaction terminated by boiling or other form of denaturation. Released [ $^3\text{H}$ ]acetate is extracted and counted. For example, the quenched reaction mixture can be acidified with concentrated HCl, and used to create a biphasic mixture with ethyl acetate. The resulting 2 phase system is thoroughly mixed, centrifuged, and the ethyl acetate phase collected and counted by standard scintillation methods. Other methods for detecting acetate release will be easily recognized by those skilled in the art.

In yet another embodiment, the drug screening assay is derived to include a whole cell recombinantly expressing one or more of a target protein or *HDx* protein. The ability of a test agent to alter the activity of the *HDx* protein can be detected by analysis of the recombinant cell. For example, agonists and antagonists of the *HDx* biological activity can be detected by scoring for alterations in growth or differentiation (phenotype) of the cell. General techniques for detecting each are well known, and will vary with respect to the source of the particular reagent cell utilized in any given assay.

For example, quantification of proliferation of cells in the presence and absence of a candidate agent can be measured with a number of techniques well known in the art, including simple measurement of population growth curves. For instance, where the assay involves proliferation in a liquid medium, turbidimetric techniques (i.e. absorbance/transmittance of light of a given wavelength through the sample) can be utilized. For example, in the instance where the reagent cell is a yeast cell, measurement of absorbance of light at a wavelength between 540 and 600nm can provide a conveniently fast measure of cell growth. Likewise, ability to form colonies in solid medium (e.g. agar) can be used to readily score for proliferation. In other embodiments, an *HDx* substrate protein, such as a histone, can be provided as a fusion protein which permits the substrate to be isolated from cell lysates and the degree of acetylation detected. Each of these techniques are suitable for high through-put analysis necessary for rapid screening of large numbers of candidate agents.

In addition, where the ability of an agent to cause or reverse a transformed phenotype, growth in solid media such as agar can further aid in establishing whether a mammalian cell is transformed.

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Additionally, visual inspection of the morphology of the reagent cell can be used to determine whether the biological activity of the targeted *HDx* protein has been affected by the added agent. To illustrate, the ability of an agent to influence an apoptotic phenotype which is mediated in some way by a recombinant *HDx* protein can be assessed  
5 by visual microscopy. Likewise, the formation of certain cellular structures as part of differentiation, such as the formation of neuritic process, can be visualized under a light microscope.

The nature of the effect of test agent on reagent cell can be assessed by measuring levels of expression of specific genes, e.g., by reverse transcription-PCR. Another  
10 method of scoring for effect on *Hdx* activity is by detecting cell-type specific marker expression through immunofluorescent staining. Many such markers are known in the art, and antibodies are readily available. For example, the presence of chondroitin sulphate proteoglycans as well as type-II collagen are correlated with cartilage production in chondrocytes, and each can be detected by immunostaining. Similarly, the  
15 human kidney differentiation antigen gp160, human aminopeptidase A, is a marker of kidney induction, and the cytoskeletal protein troponin I is a marker of heart induction. In yet another embodiment, the alteration of expression of a reporter gene construct provided in the reagent cell provides a means of detecting the effect on *HDx* activity. For example, reporter gene constructs derived using the transcriptional regulatory sequences,  
20 e.g. the promoters, for developmentally regulated genes can be used to drive the expression of a detectable marker, such as a luciferase gene. In an illustrative embodiment, the construct is derived using the promoter sequence from a gene expressed in a particular differentiative phenotype.

It is also deemed to be within the scope of this invention that the recombinant  
25 *HDx* cells of the present assay can be generated so as to comprise heterologous *HDx* proteins (i.e. cross-species expression). For example, *HDx* proteins from one species can be expressed in the cells of another under conditions wherein the heterologous protein is able to rescue loss-of-function mutations in the host cell. For example, the reagent cell can be a yeast cell in which a human *MDx* protein (e.g. exogenously expressed) is the  
30 intended target for development of an anti-proliferative agent. To illustrate, the M778 strain, *MATa ura3-52 trp1Δ1 his3-200 leu2-1 trk1Δ rpd3Δ::HIS3*, described by Vidal et al. (1991) *Mol Cell Biol* 6317-6327, which lacks a functional endogenous RPD3 gene can be transfected with an expression plasmid including a mammalian *HDx* gene in order to complement the RPD3 loss-of-function. For example, the coding sequence for *HD1*  
35 can be cloned into a pRS integrative plasmid containing a selectable marker (Sikorski et

al. (1989) *Genetics* 122:19-27), and resulting construct used to transform the M778 strain. The resulting cells should produce a mammalian *HD1* protein which may be capable performing at least some of the functions of the yeast RPD3 protein. The *HDx* transformed yeast cells can be easier to manipulate than mammalian cells, and can provide  
5 access to certain assay formats, such as turbidity detection methods, which may not be obtainable with mammalian cells.

Moreover, the combination of the "mammalianized" strain with the strain M537 (*MATa ura3-52 trp1Δ1 his3-200 leu2-1 trk1Δ*, Vidal et al., *supra*) can provide an exquisitely sensitive cell-based assay for detecting agent which specifically inhibit, for  
10 example, the yeast RPD3 deacetylase.

In another aspect, the invention provides compounds useful for inhibition of *HDxs*. In a preferred embodiment, an *HDx* inhibitor compound of the invention can be represented by the formula A-B-C, in which A is a specificity element for selective binding to an *HDx*, B is a linker element, and C is an electrophilic moiety capable of  
15 reacting with a nucleophilic moiety of an *HDx*; with the proviso that the compound is not butyrate, trapoxin, or trichostatin.

In another aspect, the invention provides an affinity matrix for binding or purifying an *HDx*. In a preferred embodiment, the affinity matrix can be represented by the formula S-A-B-C, in which S is a solid or insoluble support, and A, B, and C are as  
20 described above. The solid or insoluble support S can be any of a variety of supports, many of which are known in the art, for synthesis of, or immobilization of, compounds, e.g., peptides, benzodiazepines, and the like. For a review of solid-supported synthesis, see, e.g., Hodge et al., *Polymer-supported Reactions in Organic Synthesis*, John Wiley & Sons, New York, 1980. The *HDx* inhibitor moiety A-B-C can be bonded directly to  
25 the support S, or can be bonded to the support S through a linking or spacing moiety, as is known in the art.

In another aspect, the invention provides a method of inhibiting an *HDx*. The method comprises contacting the *HDx* with a compound capable of inhibiting *HDx* activity, under conditions such that *HDx* activity is inhibited. In preferred embodiments,  
30 the compounds can be represented by the formula A-B-C, in which A, B, and C are as described above; with the proviso that the compound is not butyrate, trapoxin, or trichostatin.

In another aspect, the invention provides a method of purifying an *HDx*. The method includes contacting a reaction mixture comprising an *HDx* with an affinity matrix

capable of selectively binding to an *HDx*, and separating at least one other component of the reaction mixture from the *HDx*. In a preferred embodiment, the affinity matrix can be represented by the formula S-A-B-C, in which S, A, B, and C are as described above.

5 In general, the elements A, B, and C of the inhibitor compounds are selected to permit selective binding to, and inhibition of, at least one *HDx*. The elements A, B, and C can be selected to provide specificity for particular *HDxs*. For example, a series of candidate *HDx* inhibitor compounds can be synthesized, e.g., according to the combinatorial methods described *infra*, and the library of candidate compounds screened against one or more *HDxs* to determine the compound or compounds with optimal  
10 activity and specificity for a particular *HDx*.

Thus, in preferred embodiments, the specificity element A is selected such that the *HDx* inhibitor compound binds selectively to an *HDx*. In general, the specificity element A will be selected according to factors such as the binding specificity of the *HDx* or *HDxs* to which the inhibitor compound should bind, ease of synthesis, stability *in vivo* or *in*  
15 *vitro*, and the like. In certain embodiments, the specificity element A is a cyclotetrapeptidyl moiety. In another embodiment, A is a substituted or unsubstituted aryl moiety. In yet another embodiment, A is a nonaromatic carbocycle. In still another embodiment, A is an amino acyl moiety (e.g., a natural or non-natural amino acyl moiety). In yet another embodiment, A is a heterocyclyl moiety.

20 In preferred embodiments, B is selected from the group consisting of substituted and unsubstituted C<sub>4</sub>-C<sub>8</sub> alkylidene, C<sub>4</sub>-C<sub>8</sub> alkenylidene, C<sub>4</sub>-C<sub>8</sub> alkynylidene, and D-E-F, in which D and F are independently absent or C<sub>2</sub>-C<sub>7</sub> alkylidene, C<sub>2</sub>-C<sub>7</sub> alkenylidene, or C<sub>2</sub>-C<sub>7</sub> alkynylidene, and E is O, S, or NR', in which R' is H, lower alkyl, lower alkenyl, lower alkynyl, aralkyl, aryl, or heterocyclyl. The element B should be selected to permit  
25 the specificity element A to interact with an *HDx* such that specific binding occurs, while poisoning the electrophilic moiety C for reaction with a nucleophilic moiety of the *HDx*.

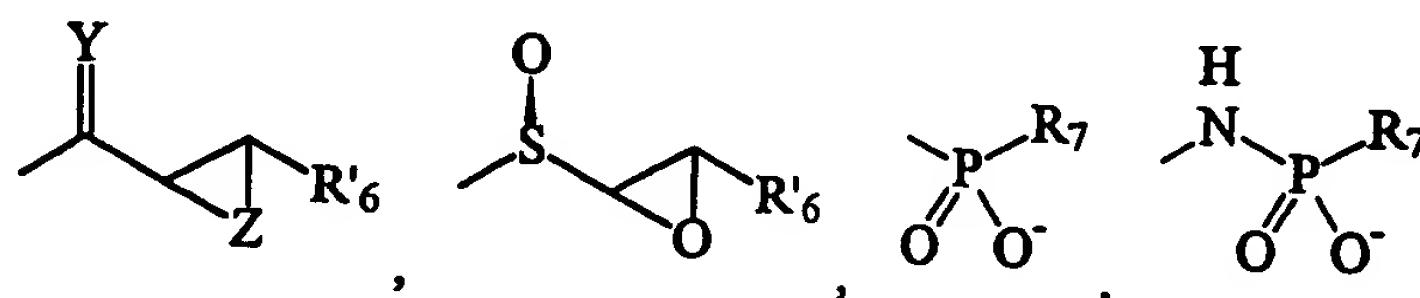
In a preferred embodiment, C is an electrophilic moiety that is approximately isosteric with an N-acetyl group (i.e., C has approximately the same steric bulk as an N-acetyl group). In preferred embodiments, the element C is capable of reacting, covalently  
30 or non-covalently, with a nucleophilic moiety of an *HDx*. In certain preferred embodiments, the element C is capable of binding (e.g., by chelation) to a metal ion, e.g., a divalent metal ion, e.g., zinc or calcium. In preferred embodiments, C is selected from the group consisting of  $\alpha,\beta$ -epoxyketones,  $\alpha,\beta$ -epoxythioketones,  $\alpha,\beta$ -epoxysulfoxides, hydroxamic acids,  $\alpha$ -haloketones,  $\alpha$ -halothioketones,  $\alpha$ -diazoketones,  $\alpha$ -

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diazothiketones, vinyl epoxides, trifluoromethylketone, trifluoromethylthioketone, enones (e.g., of ketones or thioketones), ynones (e.g., of ketones or thioketones),  $\alpha,\beta$ -aziridinoketones, hydrazones, boronic acids, carboxylates, amides (e.g., -C(O)-amino), sulfones, aldehyde, alkyl halides, epoxides, and the like.

5 In accordance with the foregoing, the moieties A, B, and C can illustratively be represented by the formulas depicted in Figure 6, in which  $R_1$  represents one or more substituents selected from the group consisting of amino, halogen, alkyl, alkenyl, alkynyl, aryl, aralkyl, heterocyclyl, azido, carboxyl, alkoxy, cyano, trifluoromethyl, and the like;  $R''$  is  $C_1$ - $C_8$  alkylidene,  $C_2$ - $C_8$  alkenylidene, or  $C_2$ - $C_8$  alkynylidene;  $R_5$  is hydrogen, alkyl, alkoxy, aryloxy, halogen, and the like;  $R'_6$  is hydrogen, alkyl, aryl, alkoxy, aryloxy, halogen, and the like;  $R'_7$  is hydrogen, alkyl, alkenyl, alkynyl, aryl, and the like;  $R_8$  is hydrogen, alkyl, halogen, and the like;  $R_9$  is hydrogen, alkyl, aryl, hydroxyl, alkoxy, aryloxy, amino, and the like; X is a good leaving group, e.g., diazo, halogen, a sulfate or sulfonate ester, e.g., a tosylate or mesylate, and the like; and Y is O or S.

In certain preferred embodiments, an *HDx* inhibitor compound can be represented by the formula A-B-C, in which A is selected from the group consisting of cycloalkyls, unsubstituted and substituted aryls, heterocyclyls, amino acyls, and cyclotetrapeptides; B is selected from the group consisting of substituted and unsubstituted  $C_4$ - $C_8$  alkylidene,  $C_4$ - $C_8$  alkenylidene,  $C_4$ - $C_8$  alkynylidene,  $C_4$ - $C_8$  enyne, and D-E-F, in which D and F are independently absent or a  $C$ - $C_7$  alkylidene, an  $C_2$ - $C_7$  alkenylidene, or an  $C_2$ - $C_7$  alkynylidene, and E is O, S, or  $NR'$ , in which  $R'$  represents H, a lower alkyl, a lower alkenyl, a lower alkynyl, an aralkyl, aryl, or a heterocyclyl; and C is selected from the group consisting of



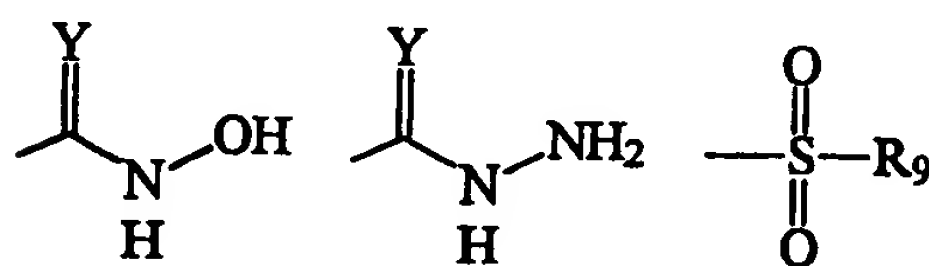
and  $B(OH)_2$  (boronic acid); in which Z represents O, S, or  $NR_5$ , and Y,  $R_5$ ,  $R'_6$ , and  $R_7$  are as defined above. In preferred embodiments,  $R'_6$  is hydrogen. In certain preferred embodiments, B is not a  $C_4$ - $C_8$  alkylidene. In preferred embodiments, if B is a  $C_4$ - $C_8$  alkylidene, C is not a boronic acid. In other preferred embodiments, the inhibitor compound is not trapoxin.

In certain preferred embodiments, an *HDx* inhibitor compound can be represented by the formula A-B-C, in which A is selected from the group consisting of cycloalkyls,



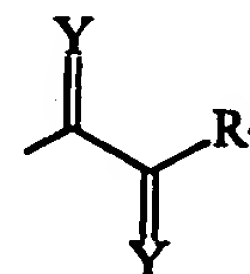
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unsubstituted and substituted aryls, heterocycllys, amino acyls, and cyclotetrapeptides; B is selected from the group consisting of substituted and unsubstituted C<sub>4</sub>-C<sub>8</sub> alkylidene, C<sub>4</sub>-C<sub>8</sub> alkenylidene, C<sub>4</sub>-C<sub>8</sub> alkynylidene, C<sub>4</sub>-C<sub>8</sub> enyne, and D-E-F, in which D and F are independently absent or C<sub>1</sub>-C<sub>7</sub> alkylidene, C<sub>2</sub>-C<sub>7</sub> alkenylidene, or C<sub>2</sub>-C<sub>7</sub> alkynylidene, and E is O, S, or NR', in which R' represents H, a lower alkyl, a lower alkenyl, a lower alkynyl, an aralkyl, an aryl, or a heterocycllyl; and C is selected from the group consisting of



, in which R<sub>9</sub> is as defined above. In preferred embodiments, B is not a C<sub>4</sub>-C<sub>8</sub> alkylidene. In preferred embodiments, the inhibitor compound is not trichostatin.

In still another preferred embodiment, an HDx inhibitor compound can be represented by the formula A-B-C, in which A is selected from the group consisting of cycloalkyls, unsubstituted and substituted aryls, heterocycllys, amino acyls, and cyclotetrapeptides; B is selected from the group consisting of substituted and unsubstituted C<sub>4</sub>-C<sub>8</sub> alkylidene, C<sub>4</sub>-C<sub>8</sub> alkenylidene, C<sub>4</sub>-C<sub>8</sub> alkynylidene, C<sub>4</sub>-C<sub>8</sub> enyne, and D-E-F, in which D and F are independently absent or a C<sub>1</sub>-C<sub>7</sub> alkylidene, a C<sub>2</sub>-C<sub>7</sub> alkenylidene, or a C<sub>2</sub>-C<sub>7</sub> alkynylidene, and E is O, S, or NR', in which R' is H, lower



alkyl, lower alkenyl, lower alkynyl, aralkyl, aryl, or heterocycllyl; and C is which Y is O or S, and R<sub>7</sub> is as defined above.

Certain HDx inhibitor compounds of the present invention may exist in particular geometric or stereoisomeric forms. For example, amino acids can contain at least one chiral center. The present invention contemplates all such compounds, including cis- and trans-isomers, R- and S-enantiomers, diastereomers, the racemic mixtures thereof, and other mixtures thereof, as falling within the scope of the invention. Additional asymmetric carbon atoms may be present in a substituent such as an alkyl group. All such isomers, as well as mixtures thereof, are intended to be included in this invention.

If, for instance, a particular enantiomer of a compound of the present invention is desired, it may be prepared by asymmetric synthesis, or by derivatization with a chiral auxiliary, where the resulting diastereomeric mixture is separated and the auxiliary group

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cleaved to provide the pure desired enantiomer. Alternatively, where the molecule contains a basic functional group, such as amino, or an acidic functional group, such as carboxyl, diastereomeric salts can be formed with an appropriate optically-active acid or base, followed by resolution of the diastereomers thus formed by fractional crystallization or chromatographic means well known in the art, and subsequent recovery of the pure enantiomers.

The term "alkyl" refers to the radical of saturated aliphatic groups, including straight-chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl substituted cycloalkyl groups, and cycloalkyl substituted alkyl groups. In preferred embodiments, a straight chain or branched chain alkyl has 30 or fewer carbon atoms in its backbone (e.g., C<sub>1</sub>-C<sub>30</sub> for straight chain, C<sub>3</sub>-C<sub>30</sub> for branched chain), and more preferably 20 or fewer. Likewise, preferred cycloalkyls have from 4-10 carbon atoms in their ring structure, and more preferably have 5, 6 or 7 carbons in the ring structure.

Unless the number of carbons is otherwise specified, "lower alkyl" as used herein means an alkyl group, as defined above, but having from one to ten carbons, more preferably from one to six carbon atoms in its backbone structure. Likewise, "lower alkenyl" and "lower alkynyl" have similar chain lengths. Preferred alkyl groups are lower alkyls. In preferred embodiments, a substituent designated herein as alkyl is a lower alkyl.

Moreover, the term "alkyl" (or "lower alkyl") as used throughout the specification and claims is intended to include both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, halogen, hydroxyl, carbonyl (such as a carboxylate, alkoxycarbonyl, aryloxy carbonyl, alkylcarbonyl, arylcarbonyl, aldehyde, and the like), thiocarbonyl (such as a thioacid, alkoxycarbonyl, and the like), an alkoxyl, unsubstituted amino, mono- or disubstituted amino, amido, amidine, imine, nitro, azido, sulfhydryl, alkylthio, cyano, trifluoromethyl, sulfonato, sulfamoyl, sulfonamido, heterocyclyl, aralkyl, or an aromatic or heteroaromatic moiety. It will be understood by those skilled in the art that the moieties substituted on the hydrocarbon chain can themselves be substituted, as described above, if appropriate. Exemplary substituted alkyls are described below. Cycloalkyls can be further substituted with, e.g., alkyls, alkenyls, alkoxys, alkylthios, aminoalkyls, carbonyl-substituted alkyls, -CF<sub>3</sub>, -CN, and the like.

The terms "alkenyl" and "alkynyl" refer to unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double or triple bond respectively. The term "enyne" refers to an unsaturated aliphatic moiety having at least one double bond and one triple bond.

- 5       The terms "alkylidene," "alkenylidene," and "alkynylidene" are art-recognized and refer to moieties corresponding to alkyl, alkenyl, and alkynyl moieties as defined above, but having two valences available for bonding.

- 10       The term "aryl" as used herein includes 5-, 6- and 7-membered single-ring aromatic groups that may include from zero to four heteroatoms, for example, phenyl, pyrrolyl, furanyl, thiophenyl, imidazolyl, oxazolyl, thiazolyl, triazolyl, pyrazolyl, pyridyl, pyrazinyl, pyridazinyl and pyrimidyl, and the like. Those aryl groups having heteroatoms in the ring structure may also be referred to as "aryl heterocycles" or "heteroaromatics". The aromatic ring can be substituted at one or more ring positions with such substituents as described above, as for example, halogen, azido, alkyl, aralkyl, alkenyl, alkynyl, 15 cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, sulfonamido, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, -CF<sub>3</sub>, -CN, or the like.

The term "aralkyl", as used herein, refers to an alkyl group substituted with an aryl group (e.g., an aromatic or heteroaromatic group).

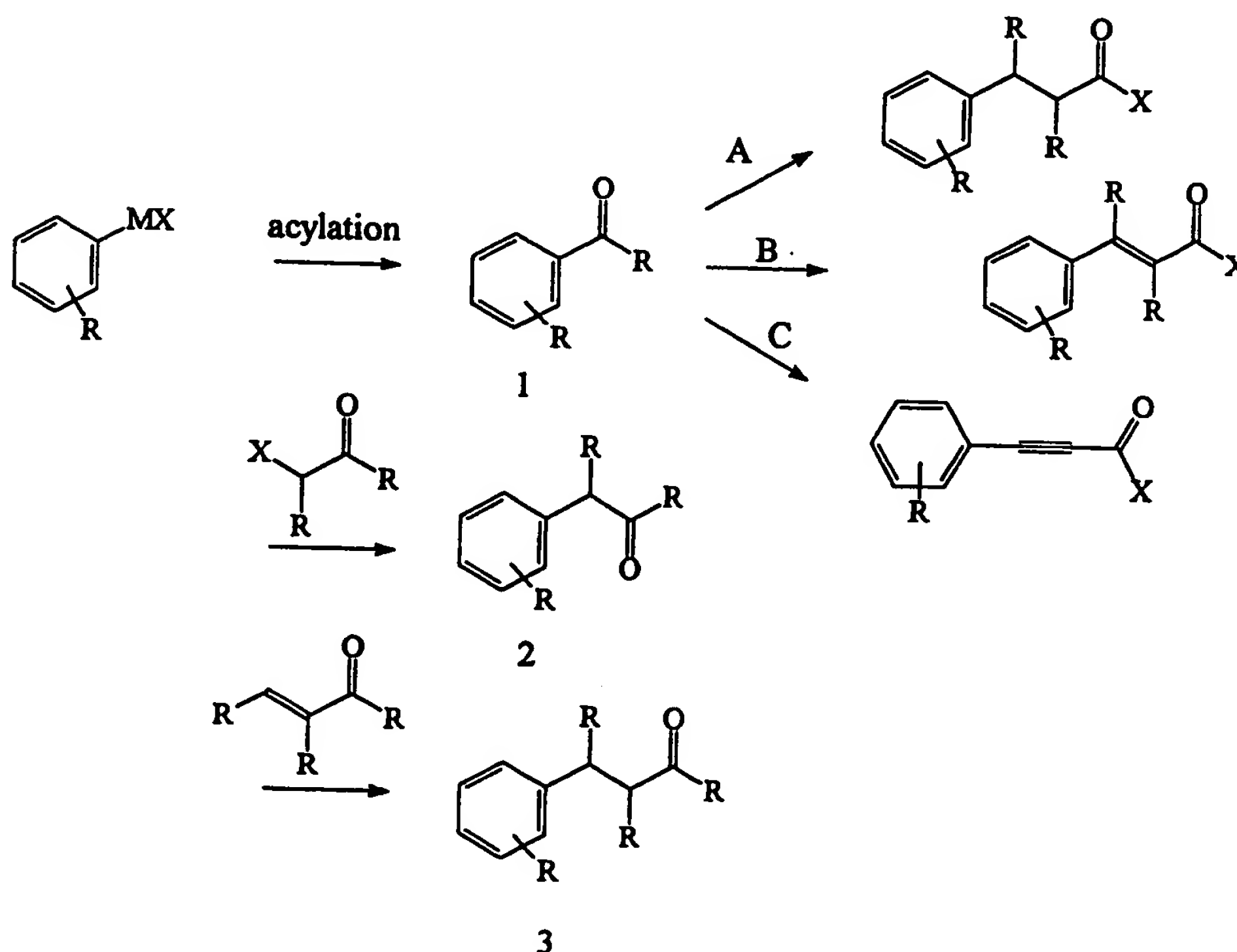
- 20       The terms "heterocyclyl" or "heterocyclic group" refer to non-aromatic 4- to 10-membered ring structures, more preferably 4- to 7-membered rings, which ring structures include one to four heteroatoms (e.g., O, N, S, P and the like). Heterocyclyl groups include, for example, pyrrolidine, oxolane, thiolane, imidazole, oxazole, piperidine, piperazine, morpholine, lactones, lactams such as azetidinones and pyrrolidinones, 25 sultams, sultones, and the like. The heterocyclic ring can be substituted at one or more positions with such substituents as described above, as for example, halogen, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, alkoxycarbonyl, aryloxycarbonyl, carboxyl, silyl, ether, alkylthio, alkylsulfonyl, arylsulfonyl, ketone (e.g., -C(O)-alkyl or -C(O)-aryl), aldehyde, heterocyclyl, an aryl or 30 heteroaryl moiety, -CF<sub>3</sub>, -CN, or the like.

Compounds represented by the formula A-B-C, in which A, B, and C have the values described *supra*, can be synthesized by standard techniques of organic synthesis. For example, precursor synthons corresponding to each of the moieties A, B, and C, or subunits thereof, can be coupled in linear or convergent syntheses to provide HDx

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inhibitor compounds, or compounds readily converted thereto. Syntheses of the HDx inhibitor compound trichostatin, and related compounds, have been reported; see, e.g., Massa, S. *et al.* (1990) *J. Med. Chem.* 33:2845-49; Mori, K., and Kosecki, K. (1988) *Tetrahedron* 44:6013-20; Koseki, K. and Mori, K. European Patent Application EP 331524 A2; Fleming, I. *et al.* (1983) *Tetrahedron* 39:841-46. Analogs of trapoxin have also been synthesized; see, e.g., Yoshida, H. and Sugita, K. (1992) *Jpn. J. Cancer Res.* 83:324-28.

Thus, in an illustrative synthesis, a compound represented by the formula A-B-C, in which A is an phenyl group, while B and C can have a variety of values, can be synthesized as shown below:



Scheme I

According to the Scheme, a functionalized organometallic aryl compound (MX=organometallic moiety; R is any substituent; X is a leaving group, e.g., halogen) (e.g., organotin, boronate, aryllithium, cuprate, Grignard reagent, etc.) is alkylated or acylated to provide functionalized compounds (e.g., the exemplary compounds 1, 2, or 3)

which can be further elaborated to provide compounds with a wide variety of substituents and carbon backbones. Other A moieties (e.g., specificity elements) can be obtained by use of appropriate synthons, e.g., by substituting vinylorganometallic compounds for the organometallic aryl compound of the Scheme (followed by further treatment, e.g.,  
5 reduction, of the vinyl group, if desired, to yield an alkyl A moiety). By way of illustration, as shown for compound 1, the carbonyl group can be used for elaboration, e.g., by reduction of the carbonyl group to an alcohol, conversion of the alcohol to a tosylate, and nucleophilic displacement of the tosylate by an acyl compound (e.g., a ketone or ester) to provide a chain-lengthened product (Route A), which can be  
10 converted to a C(O)X functionality (e.g., by hydrolysis of an ester and conversion of the resulting carboxylic acid to an acid chloride). Alternatively, the carbonyl group of 1 can be used for olefination (Route B), e.g., Horner-Emmons olefination, to provide an elaborated alkenyl compound. Also, the carbonyl group can be converted to an alkynyl functionality, e.g., via the Corey-Fuchs procedure, to provide an elaborated alkynyl  
15 compound. For purposes of clarity, only certain chain lengths and functional group patterns are shown in the scheme; however, the skilled artisan will appreciate that many other compounds, with a variety of B moieties (i.e., linking moieties), can be synthesized through analogous procedures. The C(O)X functionality (e.g., an acid chloride where X is Cl) can be converted to functional groups such as amide, hydrazido, trifluormethylketone, enone, epoxide, aziridine, and the like, through methods  
20 conventional in the art. Thus, the synthetic pathways shown in the Scheme provide access to compounds having a variety of C moieties (e.g., reactive moieties) suitable for substitution in the subject HDx inhibitors.

*In vitro* chemical synthesis provides a method for generating libraries of  
25 compounds that can be screened for ability to bind to or inhibit a target protein, e.g., an HDx. Although *in vitro* methods have previously been used in the pharmaceutical industry to identify potential drugs, recently developed methods have focused on rapidly and efficiently generating and screening large numbers of compounds and are amenable to generating HDx inhibitor compound libraries for use in the subject method. The various  
30 approaches to simultaneous preparation and analysis of large numbers of compounds (herein "combinatorial synthesis") each rely on the fundamental concept of synthesis on a solid support introduced for peptides by Merrifield in 1963 (Merrifield, R.B. (1963) *J Am Chem Soc* 85:2149-2154). Many types of solid matrices have been successfully used in solid-phase synthesis, and can be selected according to the type of chemistry to be  
35 performed on the immobilized moieties, as is discussed in more detail below.



Several synthetic schemes have been suggested or employed for the combinatorial synthesis of organic compounds (see, e.g., E.M. Gordon *et al.*, *J. Med. Chem.* **37**:1385-1401 (1994)).

## 5 Multipin Synthesis

One method for combinatorial synthesis of compounds is the multipin synthesis method. Briefly, Geysen and co-workers (Geysen *et al.* (1984) *PNAS* **81**:3998-4002) introduced a method for generating compounds by a parallel synthesis on polyacrylic acid-grated polyethylene pins arrayed in the microtitre plate format. In the original experiments, about 50 nmol of a single compound was covalently linked to the spherical head of each pin, and interactions of each compound with a receptor or antibody could be determined in a direct binding assay. The Geysen technique can be used to synthesize and screen thousands of compounds per week using the multipin method, and the tethered compounds may be reused in many assays. In subsequent work, the level of compound loading on individual pins has been increased to as much as 2  $\mu$ mol/pin by grafting greater amounts of functionalized acrylate derivatives to detachable pin heads, and the size of the compound library has been increased (Valerio *et al.* (1993) *Int J Pept Protein Res* **42**:1-9). Appropriate linker moieties have also been appended to the pins so that the compounds may be cleaved from the supports after synthesis for assessment of purity and evaluation in competition binding or functional bioassays (Bray *et al.* (1990) *Tetrahedron Lett* **31**:5811-5814; Valerio *et al.* (1991) *Anal Biochem* **197**:168-177; Bray *et al.* (1991) *Tetrahedron Lett* **32**:6163-6166).

More recent applications of the multipin method have taken advantage of the cleavable linker strategy to prepare soluble compound libraries (Maeji *et al.* (1990) *J Immunol Methods* **134**:23-33; Gammon *et al.* (1991) *J Exp Med* **173**:609-617; Mutch *et al.* (1991) *Pept Res* **4**:132-137).

## Divide-Couple-Recombine

In another embodiment, a variegated library of HDx inhibitor compounds is provided on a set of beads utilizing the strategy of divide-couple-recombine (see, e.g., Houghten (1985) *PNAS* **82**:5131-5135; and U.S. Patents 4,631,211; 5,440,016; 5,480,971). Briefly, as the name implies, at each synthesis step where degeneracy (e.g., a plurality of different moieties) is introduced into the library, the beads are divided into as many separate groups to correspond to the number of different residues (e.g., functional

groups or other moieties) to be added at that position, the different residues coupled in separate reactions, and the beads recombined into one pool for the next step.

In one embodiment, the divide-couple-recombine strategy can be carried out using the so-called "tea bag" method first developed by Houghten, where synthesis occurs on resin that is sealed inside porous polypropylene bags (Houghten et al. (1986) *PNAS* 82:5131-5135). Residues are coupled to the resins by placing the bags in solutions of the appropriate individual activated monomers, while all common steps such as resin washing and deprotection (if appropriate) are performed simultaneously in one reaction vessel. At the end of the synthesis, each bag contains a single compound, and the compounds may be liberated from the resins using a multiple cleavage apparatus (Houghten et al. (1986) *Int J Pept Protein Res* 27:673-678). This technique offers advantages of considerable synthetic flexibility and has been partially automated (Beck-Sickinger et al. (1991) *Pept Res* 4:88-94). Moreover, compounds can be produced in sufficient quantities ( $> 500 \mu\text{mol}$ ) for purification and complete characterization if desired.

Synthesis using the tea-bag approach is useful for the production of a library, albeit of limited size, as is illustrated by its use in a range of molecular recognition problems including antibody epitope analysis (Houghten et al. (1986) *PNAS* 82:5131-5135), peptide hormone structure-function studies (Beck-Sickinger et al. (1990) *Int J Pept Protein Res* 36:522-530; Beck-Sickinger et al. (1990) *Eur J Biochem* 194:449-456), and protein conformational mapping (Zimmerman et al. (1991) *Eur J Biochem* 200:519-528).

## 25 *Combinatorial Synthesis on Nontraditional Solid Supports*

The search for innovative methods of solid-phase synthesis has led to the investigation of alternative polymeric supports to the polystyrene-divinylbenzene matrix originally popularized by Merrifield. Cellulose, either in the form of paper disks (Blankemeyer-Menge et al. (1988) *Tetrahedron Lett* 29:5871-5874; Frank et al. (1988) *Tetrahedron* 44:6031-6040; Eichler et al. (1989) *Collect Czech Chem Commun* 54:1746-1752; Frank, R. (1993) *Bioorg Med Chem Lett* 3:425-430) or cotton fragments (Eichler et al. (1991) *Pept Res* 4:296-307; Schmidt et al. (1993) *Bioorg Med Chem Lett* 3:441-446) has been successfully functionalized for peptide synthesis. Typical loadings attained with cellulose paper range from 1 to  $3 \mu\text{mol}/\text{cm}^2$ , and HPLC analysis of material cleaved from these supports indicates a reasonable quality for the synthesized peptides.

Alternatively, peptides may be synthesized on cellulose sheets via non-cleavable linkers and then used in ELISA-based binding studies (Frank, R. (1992) *Tetrahedron* 48:9217-9232). The porous, polar nature of this support may help suppress unwanted nonspecific protein binding effects. In one convenient configuration synthesis occurs in an 8 x 12 microtiter plate format. Frank has used this technique to map the dominant epitopes of an antiserum raised against a human cytomegalovirus protein, following the overlapping peptide screening (Pepscan) strategy of Geysen (Frank, R. (1992) *Tetrahedron* 48:9217-9232). Other membrane-like supports that may be used for solid-phase synthesis include polystyrene-grafted polyethylene films (Berg et al. (1989) *J Am Chem Soc* 111:8024-8026).

*Combinatorial Libraries by Light-Directed, Spatially Addressable Parallel Chemical Synthesis*

A scheme of combinatorial synthesis in which the identity of a compound is given by its locations on a synthesis substrate is termed a spatially-addressable synthesis. In one embodiment, the combinatorial process is carried out by controlling the addition of a chemical reagent to specific locations on a solid support (Dower et al. (1991) *Annu Rep Med Chem* 26:271-280; Fodor, S.P.A. (1991) *Science* 251:767; Pirrung et al. (1992) U.S. Patent No. 5,143,854; Jacobs et al. (1994) *Trends Biotechnol* 12:19-26). The technique combines two well-developed technologies: solid-phase synthesis chemistry and photolithography. The high coupling yields of solid-phase reactions allows efficient compound synthesis, and the spatial resolution of photolithography affords miniaturization. The merging of these two technologies is done through the use of photolabile protecting groups, e.g., amino protecting groups, in the synthetic procedure.

The key points of this technology are illustrated in Gallop et al. (1994) *J Med Chem* 37:1233-1251. A synthesis substrate is prepared for compound synthesis through the covalent attachment of photolabile nitroveratryloxycarbonyl (NVOC) protected amino linkers. Light is used to selectively activate a specified region of the synthesis support for coupling. Removal of the photolabile protecting groups by lights (deprotection) results in activation of selected areas. After activation, the first of a set of residues, each bearing a photolabile protecting group, is exposed to the entire surface. Coupling only occurs in regions that were addressed by light in the preceding step. The reagent solution is removed, and the substrate is again illuminated through a second mask, activating a different region for reaction with a second protected building block. The pattern of masks and the sequence of reactants define the products and their locations. Since this process utilizes photolithography techniques, the number of

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compounds that can be synthesized is limited only by the number of synthesis sites that can be addressed with appropriate resolution. The position of each compound is precisely known; hence, its interactions with other molecules can be directly assessed. The target can be labeled with a fluorescent reporter group to facilitate the identification of specific interactions with individual members of the matrix.

In a light-directed chemical synthesis, the products depend on the pattern of illumination and on the order of addition of reactants. By varying the lithographic patterns, many different sets of test compounds can be synthesized in the same number of steps; this leads to the generation of many different masking strategies.

#### *Encoded Combinatorial Libraries*

In yet another embodiment, the subject method provides an *HDx* inhibitor compound library provided with an encoded tagging system. A recent improvement in the identification of active compounds from combinatorial libraries employs chemical indexing systems using tags that uniquely encode the reaction steps a given bead has undergone and, by inference, the structure it carries. Conceptually, this approach mimics phage display libraries, where activity derives from expressed peptides, but the structures of the active peptides are deduced from the corresponding genomic DNA sequence. The first encoding of synthetic combinatorial libraries employed DNA as the code. Two forms of encoding have been reported: encoding with sequenceable bio-oligomers (e.g., oligonucleotides and peptides), and binary encoding with non-sequenceable tags.

#### *Tagging with sequenceable bio-oligomers*

The principle of using oligonucleotides to encode combinatorial synthetic libraries was described in 1992 (Brenner et al. (1992) *PNAS* 89:5381-5383), and an example of such a library appeared the following year (Needles et al. (1993) *PNAS* 90:10700-10704). A combinatorial library of nominally  $7^7$  (= 823,543) peptides composed of all combinations of Arg, Gln, Phe, Lys, Val, D-Val and Thr (three-letter amino acid code), each of which was encoded by a specific dinucleotide (TA, TC, CT, AT, TT, CA and AC, respectively), was prepared by a series of alternating rounds of peptide and oligonucleotide synthesis on solid support. In this work, the amine linking functionality on the bead was specifically differentiated toward peptide or oligonucleotide synthesis by simultaneously preincubating the beads with reagents that generate protected OH groups for oligonucleotide synthesis and protected  $\text{NH}_2$  groups for peptide synthesis (here, in a ratio of 1:20). When complete, the tags each consisted of 69-mers, 14 units of which carried the code. The bead-bound library was incubated with a fluorescently labeled

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antibody, and beads containing bound antibody that fluoresced strongly were harvested by fluorescence-activated cell sorting (FACS). The DNA tags were amplified by PCR and sequenced, and the predicted peptides were synthesized. Following such techniques, HDx inhibitor compound libraries can be derived and screened using HDxs of the subject  
5 invention.

It is noted that an alternative approach useful for generating nucleotide-encoded synthetic peptide libraries employs a branched linker containing selectively protected OH and NH<sub>2</sub> groups (Nielsen et al. (1993) *J Am Chem Soc* 115:9812-9813; and Nielsen et al. (1994) *Methods Compan Methods Enzymol* 6:361-371). This approach requires that  
10 equimolar quantities of test peptide and tag co-exist, though this may be a potential complication in assessing biological activity, especially with nucleic acid based targets.

The use of oligonucleotide tags permits exquisitely sensitive tag analysis. Even so, the method requires careful choice of orthogonal sets of protecting groups required for alternating co-synthesis of the tag and the library member. Furthermore, the chemical  
15 lability of the tag, particularly the phosphate and sugar anomeric linkages, may limit the choice of reagents and conditions that can be employed for the synthesis on non-oligomeric libraries. In preferred embodiments, the libraries employ linkers permitting selective detachment of the test HDx inhibitor compound library member for bioassay, in part (as described *infra*) because assays employing beads limit the choice of targets, and  
20 in part because the tags are potentially susceptible to biodegradation.

Peptides themselves have been employed as tagging molecules for combinatorial libraries. Two exemplary approaches are described in the art, both of which employ branched linkers to solid phase upon which coding and ligand strands are alternately  
25 elaborated. In the first approach (Kerr JM et al. (1993) *J Am Chem Soc* 115:2529-2531), orthogonality in synthesis is achieved by employing acid-labile protection for the coding strand and base-labile protection for the ligand strand.

In an alternative approach (Nikolaiev et al. (1993) *Pept Res* 6:161-170), branched linkers are employed so that the coding unit and the test peptide are both attached to the same functional group on the resin. In one embodiment, a linker can be placed between  
30 the branch point and the bead so that cleavage releases a molecule containing both code and ligand (Ptek et al. (1991) *Tetrahedron Lett* 32:3891-3894). In another embodiment, the linker can be placed so that the test peptide can be selectively separated from the bead, leaving the code behind. This last construct is particularly valuable because it permits screening of the test peptide without potential interference, or biodegradation, of



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the coding groups. Examples in the art of independent cleavage and sequencing of peptide library members and their corresponding tags has confirmed that the tags can accurately predict the peptide structure.

It is noted that peptide tags are more resistant to decomposition during ligand  
5 synthesis than are oligonucleotide tags, but they must be employed in molar ratios nearly equal to those of the ligand on typical 130  $\mu$ m beads in order to be successfully sequenced. As with oligonucleotide encoding, the use of peptides as tags requires complex protection/deprotection chemistries.

10 *Non-sequenceable tagging: binary encoding*

An alternative form of encoding the test peptide library employs a set of non-sequenceable tagging molecules (e.g., molecules having electrophoric moieties) that are used as a binary code (Ohlmeyer et al. (1993) *PNAS* 90:10922-10926). Exemplary tags are haloaromatic alkyl ethers that are detectable as their trimethylsilyl ethers at less than  
15 femtomolar levels by electron capture gas chromatography (ECGC). Variations in the length of the alkyl chain, as well as the nature and position of the aromatic halide substituents, permit the synthesis of at least 40 such tags, which in principle can encode  $2^{40}$  (e.g., upwards of  $10^{12}$ ) different molecules. In the original report (Ohlmeyer et al., *supra*) the tags were bound to about 1% of the available amine groups of a peptide  
20 library via a photocleavable O-nitrobenzyl linker. This approach is convenient when preparing combinatorial libraries of peptides or other amine-containing molecules. A more versatile system has, however, been developed that permits encoding of essentially any combinatorial library. Here, the ligand is attached to the solid support via the photocleavable linker and the tag is attached through a catechol ether linker via carbene  
25 insertion into the bead matrix (Nestler et al. (1994) *J Org Chem* 59:4723-4724). This orthogonal attachment strategy permits the selective detachment of library members for bioassay in solution and subsequent decoding by ECGC after oxidative detachment of the tag sets.

Binary encoding with tags, e.g., electrophoric tags, has been particularly useful in  
30 defining selective interactions of substrates with synthetic receptors (Borchardt et al. (1994) *J Am Chem Soc* 116:373-374), and model systems for understanding the binding and catalysis of biomolecules. Even using detailed molecular modeling, the identification of the selectivity preferences for synthetic receptors has required the manual synthesis of dozens of potential substrates. The use of encoded libraries makes it possible to rapidly  
35 examine all the members of a potential binding set. The use of binary-encoded libraries

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has made the determination of binding selectivities so facile that structural selectivity has been reported for four novel synthetic macrobicyclic and tricyclic receptors in a single communication (Wennemers et al. (1995) *J Org Chem* 60:1108-1109; and Yoon et al. (1994) *Tetrahedron Lett* 35:8557-8560) using the encoded library mentioned above.

5 Similar facility in defining specificity of interaction would be expected for many other biomolecules.

Although the several amide-linked libraries in the art employ binary encoding with the electrophoric tags attached to amine groups, attaching these tags directly to the bead matrix provides far greater versatility in the structures that can be prepared in encoded

10 combinatorial libraries. Attached in this way, the tags and their linker are nearly as unreactive as the bead matrix itself. Two binary-encoded combinatorial libraries have been reported where the tags are attached directly to the solid phase (Ohlmeyer et al. (1995) *PNAS* 92:6027-6031) and provide guidance for generating the subject *HDx* inhibitor compound library. Both libraries were constructed using an orthogonal

15 attachment strategy in which the library member was linked to the solid support by a photolabile linker and the tags were attached through a linker cleavable only by vigorous oxidation. Because the library members can be repetitively partially photoeluted from the solid support, library members can be utilized in multiple assays. Successive photoelution also permits a very high throughput iterative screening strategy: first, multiple beads are

20 placed in 96-well microtiter plates; second, ligands are partially detached and transferred to assay plates; third, a bioassay identifies the active wells; fourth, the corresponding beads are rearranged singly into new microtiter plates; fifth, single active compounds are identified; and sixth, the structures are decoded.

The above approach was employed in screening for carbonic anhydrase (CA)

25 binding and identified compounds which exhibited nanomolar affinities for CA. Unlike sequenceable tagging, a large number of structures can be rapidly decoded from binary-encoded libraries (a single ECGC apparatus can decode 50 structures per day). Thus, binary-encoded libraries can be used for the rapid analysis of structure-activity relationships and optimization of both potency and selectivity of an active series. The

30 synthesis and screening of large unbiased binary encoded *HDx* inhibitor compound libraries for lead identification, followed by preparation and analysis of smaller focused libraries for lead optimization, offers a particularly powerful approach to discovery of *HDx* inhibitor compounds.

*HDx* inhibitor compounds can be synthesized on solid support by appropriate

35 functionalization for attachment to a solid matrix, or alternatively, by solution-phase

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synthesis followed by immobilization through an appropriate functional group. Thus, in an illustrative embodiment, an *HDx* inhibitor compound, which is analogous to trichostatin, can be synthesized on a solid support by attachment through an amino group of the specificity element A, as shown in Figure 7. The solid support is preferably  
5 capable of withstanding synthetic conditions required to synthesize the requisite compounds. The compound can preferably be released from the solid support, e.g., by selective cleavage of an amide bond.

The synthetic steps employed to synthesize compounds on solid support are preferably selected to allow a wide variety of residues (e.g., building blocks) to be  
10 coupled to the immobilized moieties, preferably under mild conditions. Suitable reaction chemistries include well-known carbon-carbon bond forming reactions such as the Stille and Suzuki couplings, as well as Horner-Emmons reactions, Ni/Cr mediated couplings, and the like. Particularly preferred coupling reactions can be performed in the presence of water and do not require harsh conditions or expensive reagents.

15 Thus, in an exemplary synthesis shown in Figure 7, substituted N-methyl-4-(tributyltin)anilines (in which  $R_1$  represents one or more substitutions, e.g., hydrogen, halogen, alkyl, alkoxy, and the like) are coupled in a plurality of reaction vessels to beads of a solid support (e.g., Affigel). The beads are further divided into a plurality of reaction vessels, and suspended in a solvent such as DMF, and one acid chloride building block  
20 (corresponding to linking element B) is introduced into each vessel ( $R_2$  and  $R_3$  represent, e.g., hydrogen, halogen, alkyl, and the like; and the broken line represents an optional double bond). The reactions are stirred under an inert gas (e.g. nitrogen) and a palladium catalyst (e.g.,  $Pd(PPh_3)_4$ ) is added (0.1-1.0 mol%). The reaction is stirred for 1-24 hours. Upon completion of the reaction, the beads are washed, and placed in a plurality  
25 of vessels. The aldehyde moiety is deprotected by mild acid treatment (e.g., PPTS in MeOH), and the beads are again washed and placed in a plurality of reaction vessels, and the beads are suspended in dry acetonitrile. One building block (corresponding to the reactive element C) is then added to each reaction vessel. As illustratively shown in Figure 7, a plurality of phosphonates can be employed ( $R_4$  represents, e.g., alkyl, alkenyl,  
30 alkynyl, alkoxy, and the like). A Horner-Emmons reaction is performed by addition of LiCl (1.1 equiv.) and diisopropylethylamine (DIPEA) or DBU (1.2 equiv). Upon completion of the reaction, the beads are washed with water and acetonitrile, and then dried to yield a library of candidate *HDx* inhibitor compounds on solid support. The compounds can then be released from the solid support into solution; or the compounds  
35 can be screened while attached to the solid support.

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The above combinatorial synthesis can be performed in an encoded mode, e.g., the binary tagging method described *supra*, by addition of the appropriate tag for each monomer. In this mode, after each reaction has been performed and the corresponding tag attached, the beads from all reactions can be recombined and then divided into  
5 aliquots for further derivatization. This method provides the advantage of ease of handling when large libraries are to be synthesized. Regardless of the method of synthesis, the combinatorial library can be screened for activity according to known methods (see, e.g., Gordon *et al.*, *supra*).

10 In another aspect, the present invention provides pharmaceutically acceptable compositions which comprise a therapeutically-effective amount of one or more of the compounds described above, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. As described in detail below, the pharmaceutical compositions of the present invention may be specially formulated for  
15 administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes for application to the tongue; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for  
20 example, as a cream, ointment or spray applied to the skin; or (4) intravaginally or intrarectally, for example, as a pessary, cream or foam.

The phrase "therapeutically-effective amount" as used herein means that amount of a compound, material, or composition comprising a deacetylase inhibitor of the present invention which is effective for producing some desired therapeutic effect by inhibiting  
25 histone deacetylation in at least a sub-population of cells in an animal and thereby blocking the biological consequences of that event in the treated cells, at a reasonable benefit/risk ratio applicable to any medical treatment.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of  
30 sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically-acceptable carrier" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid

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filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject deacetylase inhibitor agent from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

As set out above, certain embodiments of the present deacetylase inhibitors may contain a basic functional group, such as amino or alkylamino, and are, thus, capable of forming pharmaceutically-acceptable salts with pharmaceutically-acceptable acids. The term "pharmaceutically-acceptable salts" in this respect, refers to the relatively non-toxic, inorganic and organic acid addition salts of compounds of the present invention. These salts can be prepared *in situ* during the final isolation and purification of the compounds of the invention, or by separately reacting a purified compound of the invention in its free base form with a suitable organic or inorganic acid, and isolating the salt thus formed. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, phosphate, nitrate, acetate, valerate, oleate, palmitate, stearate, laurate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphthylate, mesylate, glucoheptonate, lactobionate, and laurylsulphonate salts and the like. (See, for example, Berge et al. (1977) "Pharmaceutical Salts", *J. Pharm. Sci.* 66:1-19)

In other cases, the deacetylase inhibitory compounds of the present invention may contain one or more acidic functional groups and, thus, are capable of forming pharmaceutically-acceptable salts with pharmaceutically-acceptable bases. The term "pharmaceutically-acceptable salts" in these instances refers to the relatively non-toxic, inorganic and organic base addition salts of compounds of the present invention. These salts can likewise be prepared *in situ* during the final isolation and purification of the



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compounds, or by separately reacting the purified compound in its free acid form with a suitable base, such as the hydroxide, carbonate or bicarbonate of a pharmaceutically-acceptable metal cation, with ammonia, or with a pharmaceutically-acceptable organic primary, secondary or tertiary amine. Representative alkali or alkaline earth salts include the lithium, sodium, potassium, calcium, magnesium, and aluminum salts and the like. Representative organic amines useful for the formation of base addition salts include ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine and the like. (See, for example, Berge et al., *supra*)

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Formulations of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the deacetylase inhibitor which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 1 per cent to about ninety-nine percent of active ingredient, preferably from about 5 per cent to about 70 per cent, most preferably from about 10 per cent to about 30 per cent.

Methods of preparing these formulations or compositions include the step of bringing into association a compound of the present invention with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared

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by uniformly and intimately bringing into association a deacetylase inhibitor of the present invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations of the invention suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a compound of the present invention as an active ingredient. A deacetylase inhibitor of the present invention may also be administered as a bolus, electuary or paste.

In solid dosage forms of the invention for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically-acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered deacetylase inhibitor moistened with an inert liquid diluent.

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The tablets, and other solid dosage forms of the pharmaceutical compositions of the present invention, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

Liquid dosage forms for oral administration of the deacetylase inhibitors of the invention include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active deacetylase inhibitor, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Formulations of the pharmaceutical compositions of the invention for rectal or vaginal administration may be presented as a suppository, which may be prepared by

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mixing one or more compounds of the invention with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active deacetylase inhibitor.

Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate.

Dosage forms for the topical or transdermal administration of a deacetylase inhibitor of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically-acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The ointments, pastes, creams and gels may contain, in addition to an active deacetylase inhibitor of this invention, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to a compound of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Transdermal patches have the added advantage of providing controlled delivery of a compound of the present invention to the body. Such dosage forms can be made by dissolving or dispersing the deacetylase inhibitor in the proper medium. Absorption enhancers can also be used to increase the flux of the deacetylase inhibitor across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the deacetylase inhibitor in a polymer matrix or gel.

Ophthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of this invention.

Pharmaceutical compositions of this invention suitable for parenteral administration comprise one or more deacetylase inhibitors of the invention in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which

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may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

5        Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, 10 such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

      These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal 15 agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

20        In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. 25 Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

      Injectable depot forms are made by forming microencapsule matrices of the subject deacetylase inhibitors in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular 30 polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.



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When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (more preferably, 0.5 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

5       The preparations of the present invention may be given orally, parenterally, topically, or rectally. They are of course given by forms suitable for each administration route. For example, they are administered in tablets or capsule form, by injection, inhalation, eye lotion, ointment, suppository, etc. administration by injection, infusion or inhalation; topical by lotion or ointment; and rectal by suppositories. Oral administration  
10 is preferred.

These deacetylase inhibitor may be administered to humans and other animals for therapy by any suitable route of administration, including orally, nasally, as by, for example, a spray, rectally, intravaginally, parenterally, intracisternally and topically, as by powders, ointments or drops, including buccally and sublingually.

15       Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically-acceptable dosage forms by conventional methods known to those of skill in the art.

20       Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

25       The selected dosage level will depend upon a variety of factors including the activity of the particular deacetylase inhibitor employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular deacetylase inhibitor employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

30       A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required

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in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

Another aspect of the present invention relates to a method of inducing and/or maintaining a differentiated state, enhancing survival, and/or inhibiting (or alternatively  
5 potentiating) proliferation of a cell, by contacting the cells with an agent which modulates *HDx*-dependent transcription. For instance, it is contemplated by the invention that, in light of the present finding of an apparently broad involvement of *HDx* proteins in the control of chromatin structure and, thus, transcription and replication, the subject method could be used to generate and/or maintain an array of different tissue both *in vitro* and *in*  
10 *vivo*. An "*HDx* therapeutic," whether inhibitory or potentiating with respect to modulating histone deacetylation, can be, as appropriate, any of the preparations described above, including isolated polypeptides, gene therapy constructs, antisense molecules, peptidomimetics or agents identified in the drug assays provided herein.

The *HDx* compounds of the present invention are likely to play an important role  
15 in the modulation of cellular proliferation. There are a wide variety of pathological cell proliferative conditions for which *HDx* therapeutics of the present invention may be used in treatment. For instance, such agents can provide therapeutic benefits where the general strategy being the inhibition of an anomalous cell proliferation. Diseases that might benefit from this methodology include, but are not limited to various cancers and  
20 leukemias, psoriasis, bone diseases, fibroproliferative disorders such as involving connective tissues, atherosclerosis and other smooth muscle proliferative disorders, as well as chronic inflammation.

In addition to proliferative disorders, the present invention contemplates the use of *HDx* therapeutics for the treatment of differentiative disorders which result from, for  
25 example, de-differentiation of tissue which may (optionally) be accompanied by abortive reentry into mitosis, e.g. apoptosis. Such degenerative disorders include chronic neurodegenerative diseases of the nervous system, including Alzheimer's disease, Parkinson's disease, Huntington's chorea, amyotrophic lateral sclerosis and the like, as well as spinocerebellar degenerations. Other differentiative disorders include, for  
30 example, disorders associated with connective tissue, such as may occur due to de-differentiation of chondrocytes or osteocytes, as well as vascular disorders which involve de-differentiation of endothelial tissue and smooth muscle cells, gastric ulcers characterized by degenerative changes in glandular cells, and renal conditions marked by failure to differentiate, e.g. Wilm's tumors.

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It will also be apparent that, by transient use of modulators of *HDx* activities, *in vivo* reformation of tissue can be accomplished, e.g. in the development and maintenance of organs. By controlling the proliferative and differentiative potential for different cells, the subject *HDx* therapeutics can be used to reform injured tissue, or to improve grafting and morphology of transplanted tissue. For instance, *HDx* antagonists and agonists can be employed in a differential manner to regulate different stages of organ repair after physical, chemical or pathological insult. For example, such regimens can be utilized in repair of cartilage, increasing bone density, liver repair subsequent to a partial hepatectomy, or to promote regeneration of lung tissue in the treatment of emphysema.

10 The present method is also applicable to cell culture techniques.

In one embodiment, the *HDx* therapeutic of the present invention can be used to induce differentiation of uncommitted progenitor cells and thereby give rise to a committed progenitor cell, or to cause further restriction of the developmental fate of a committed progenitor cell towards becoming a terminally-differentiated cell. For example, the present method can be used *in vitro* or *in vivo* to induce and/or maintain the differentiation of hematopoietic cells into erythrocytes and other cells of the hematopoietic system. In an illustrative embodiment, the effect of erythropoietin (EPO) on the growth of EPO-responsive erythroid precursor cells is increased to influence their differentiation into red blood cells. For example, as a result of administering an inhibitor of histone deacetylation, the amount of EPO, or other differentiating agent, required for growth and/or differentiation is reduced (PCT/US92/07737). Accordingly, the *HDx* therapeutics of the present invention, particularly those which antagonize *HDx* deacetylase activity, can be administered alone or in conjunction with EPO and in a suitable carrier to vertebrates to promote erythropoiesis. Alternatively, cells could be treated *ex vivo*. Such treatment is contemplated in the treatment of a variety of disease states, including in individuals who require bone marrow transplants (e.g. patients with aplastic anemia, acute leukemias, recurrent lymphomas, or solid tumors).

To illustrate, prior to receiving a bone marrow transplant, a recipient is prepared by ablating or removing endogenous hematopoietic stem cells. Such treatment is usually carried out by total body irradiation or delivery of a high dose of an alkylating agent or other chemotherapeutic, cytotoxic agent, Anklesaria, et al. (1987) *PNAS* 84:7681-7685). Following preparation of the recipient, donor bone marrow cells are injected intravenously. Optionally, the *HDx* therapeutics of the present invention could be contacted with the cells *ex vivo* or administered to the subject with the reimplanted cells.

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It is also contemplated that there may be cell-type specific *HDx* proteins, and/or that some cell types may be more sensitive to modulation of *HDx* deacetylase activities. Even within a cell type, the stage of differentiation or position in the cell cycle could influence their response to an *HDx* therapeutic. Accordingly, the present invention  
5 contemplates the use of agents which modulate histone deacetylase activity to specifically inhibit or activate certain cell types. In an illustrative example, T cell proliferation could be preferentially inhibited in order to induce tolerance by using a procedure similar to that for inducing tolerance using sodium butyrate (see, for example, PCT/US93/03045). To illustrate, the *HDx* therapeutics of the present invention may be used to induce antigen-  
10 specific tolerance in any situation in which it is desirable to induce tolerance, such as autoimmune diseases, in allogeneic or xenogeneic transplant recipients, or in graft versus host (GVH) reactions. According to the invention, tolerance will typically be induced by presenting the tolerizing compound (e.g., an *HDx* inhibitor) substantially contemporaneously with the antigen, i.e. reasonably close together in time with the  
15 antigen. In preferred embodiments the *HDx* therapeutic will be administered after presentation of the antigen, so that they will have their effect after the particular repertoire of Th cells begins to undergo clonal expansion.

Yet another aspect of the present invention concerns the application of *HDx* therapeutics to modulating morphogenic signals involved in organogenic pathways.  
20 Thus, it is contemplated by the invention that compositions comprising *HDx* therapeutics can also be utilized for both cell culture and therapeutic methods involving generation and maintenance of tissue.

In a further embodiment of the invention, the subject *HDx* therapeutics will be useful in increasing the amount of protein produced by a cell or recombinant cell. The  
25 cell may include any primary cell isolated from any animal, cultured cells, immortalized cells, and established cell lines. The animal cells used in the present invention include cells which intrinsically have an ability to produce a desired protein; cells which are induced to have an ability to produce a desired protein, for example, by stimulation with a cytokine such as an interferon, an interleukin; genetically engineered cells into which a  
30 gene for a desired protein is introduced. The protein produced by the process could include any peptides or proteins, including peptide hormone or proteinaceous hormones such as any useful hormone, cytokine, interleukin, or protein which it may be desirable to have in purified form and/or in large quantity.

Another aspect of the invention features transgenic non-human animals which  
35 express a heterologous *HDx* gene of the present invention, or which have had one or

more genomic *HDx* genes disrupted in at least one of the tissue or cell-types of the animal. Accordingly, the invention features an animal model for developmental diseases, which animal has one or more *HDx* allele which is mis-expressed. For example, a mouse can be bred which has one or more *HDx* alleles deleted or otherwise rendered inactive.

5 Such a mouse model can then be used to study disorders arising from mis-expressed *HDx* genes, as well as for evaluating potential therapies for similar disorders.

Another aspect of the present invention concerns transgenic animals which are comprised of cells (of that animal) which contain a transgene of the present invention and which preferably (though optionally) express an exogenous *HDx* protein in one or more

10 cells in the animal. An *HDx* transgene can encode the wild-type form of the protein, or can encode homologs thereof, including both agonists and antagonists, as well as antisense constructs. In preferred embodiments, the expression of the transgene is restricted to specific subsets of cells, tissues or developmental stages utilizing, for example, cis-acting sequences that control expression in the desired pattern. In the

15 present invention, such mosaic expression of an *HDx* protein can be essential for many forms of lineage analysis and can additionally provide a means to assess the effects of, for example, lack of *HDx* expression which might grossly alter development in small patches of tissue within an otherwise normal embryo. Toward this end, tissue-specific regulatory sequences and conditional regulatory sequences can be used to control expression of the

20 transgene in certain spatial patterns. Moreover, temporal patterns of expression can be provided by, for example, conditional recombination systems or prokaryotic transcriptional regulatory sequences.

Genetic techniques which allow for the expression of transgenes can be regulated via site-specific genetic manipulation *in vivo* are known to those skilled in the art. For

25 instance, genetic systems are available which allow for the regulated expression of a recombinase that catalyzes the genetic recombination a target sequence. As used herein, the phrase "target sequence" refers to a nucleotide sequence that is genetically recombined by a recombinase. The target sequence is flanked by recombinase recognition sequences and is generally either excised or inverted in cells expressing

30 recombinase activity. Recombinase catalyzed recombination events can be designed such that recombination of the target sequence results in either the activation or repression of expression of one of the subject *HDx* proteins. For example, excision of a target sequence which interferes with the expression of a recombinant *HDx* gene, such as one which encodes an antagonistic homolog or an antisense transcript, can be designed to

35 activate expression of that gene. This interference with expression of the protein can



result from a variety of mechanisms, such as spatial separation of the *HDx* gene from the promoter element or an internal stop codon. Moreover, the transgene can be made wherein the coding sequence of the gene is flanked by recombinase recognition sequences and is initially transfected into cells in a 3' to 5' orientation with respect to the promoter element. In such an instance, inversion of the target sequence will reorient the subject gene by placing the 5' end of the coding sequence in an orientation with respect to the promoter element which allow for promoter driven transcriptional activation.

In an illustrative embodiment, either the cre/loxP recombinase system of bacteriophage P1 (Lakso et al. (1992) PNAS 89:6232-6236; Orban et al. (1992) PNAS 89:6861-6865) or the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman et al. (1991) Science 251:1351-1355; PCT publication WO 92/15694) can be used to generate *in vivo* site-specific genetic recombination systems. Cre recombinase catalyzes the site-specific recombination of an intervening target sequence located between loxP sequences. loxP sequences are 34 base pair nucleotide repeat sequences to which the Cre recombinase binds and are required for Cre recombinase mediated genetic recombination. The orientation of loxP sequences determines whether the intervening target sequence is excised or inverted when Cre recombinase is present (Abremski et al. (1984) J. Biol. Chem. 259:1509-1514); catalyzing the excision of the target sequence when the loxP sequences are oriented as direct repeats and catalyzes inversion of the target sequence when loxP sequences are oriented as inverted repeats.

Accordingly, genetic recombination of the target sequence is dependent on expression of the Cre recombinase. Expression of the recombinase can be regulated by promoter elements which are subject to regulatory control, e.g., tissue-specific, developmental stage-specific, inducible or repressible by externally added agents. This regulated control will result in genetic recombination of the target sequence only in cells where recombinase expression is mediated by the promoter element. Thus, the activation expression of a recombinant *HDx* protein can be regulated via control of recombinase expression.

Use of the cre/loxP recombinase system to regulate expression of a recombinant *HDx* protein requires the construction of a transgenic animal containing transgenes encoding both the Cre recombinase and the subject protein. Animals containing both the Cre recombinase and a recombinant *HDx* gene can be provided through the construction of "double" transgenic animals. A convenient method for providing such animals is to mate two transgenic animals each containing a transgene, e.g., an *HDx* gene and recombinase gene.

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One advantage derived from initially constructing transgenic animals containing an *HDx* transgene in a recombinase-mediated expressible format derives from the likelihood that the subject protein, whether agonistic or antagonistic, can be deleterious upon expression in the transgenic animal. In such an instance, a founder population, in which the subject transgene is silent in all tissues, can be propagated and maintained. Individuals of this founder population can be crossed with animals expressing the recombinase in, for example, one or more tissues and/or a desired temporal pattern. Thus, the creation of a founder population in which, for example, an antagonistic *HDx* transgene is silent will allow the study of progeny from that founder in which disruption of *HDx* mediated induction in a particular tissue or at certain developmental stages would result in, for example, a lethal phenotype.

Similar conditional transgenes can be provided using prokaryotic promoter sequences which require prokaryotic proteins to be simultaneously expressed in order to facilitate expression of the *HDx* transgene. Exemplary promoters and the corresponding trans-activating prokaryotic proteins are given in U.S. Patent No. 4,833,080.

Moreover, expression of the conditional transgenes can be induced by gene therapy-like methods wherein a gene encoding the trans-activating protein, e.g. a recombinase or a prokaryotic protein, is delivered to the tissue and caused to be expressed, such as in a cell-type specific manner. By this method, an *HDx* transgene could remain silent into adulthood until "turned on" by the introduction of the trans-activator.

In an exemplary embodiment, the "transgenic non-human animals" of the invention are produced by introducing transgenes into the germline of the non-human animal. Embryonic target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending on the stage of development of the embryonic target cell. The zygote is the best target for micro-injection. In the mouse, the male pronucleus reaches the size of approximately 20 micrometers in diameter which allows reproducible injection of 1-2pl of DNA solution. The use of zygotes as a target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host genome before the first cleavage (Brinster et al. (1985) PNAS 82:4438-4442). As a consequence, all cells of the transgenic non-human animal will carry the incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene. Microinjection of zygotes is the preferred method for incorporating transgenes in practicing the invention.

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Retroviral infection can also be used to introduce *HDx* transgenes into a non-human animal. The developing non-human embryo can be cultured *in vitro* to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Jaenich, R. (1976) PNAS 73:1260-1264). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Manipulating the Mouse Embryo, Hogan eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1986). The viral vector system used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (Jahner et al. (1985) PNAS 82:6927-6931; Van der Putten et al. (1985) PNAS 82:6148-6152). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, supra; Stewart et al. (1987) EMBO J. 6:383-388). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (Jahner et al. (1982) Nature 298:623-628). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of the cells which formed the transgenic non-human animal. Further, the founder may contain various retroviral insertions of the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line by intrauterine retroviral infection of the midgestation embryo (Jahner et al. (1982) supra).

A third type of target cell for transgene introduction is the embryonic stem cell (ES). ES cells are obtained from pre-implantation embryos cultured *in vitro* and fused with embryos (Evans et al. (1981) Nature 292:154-156; Bradley et al. (1984) Nature 309:255-258; Gossler et al. (1986) PNAS 83: 9065-9069; and Robertson et al. (1986) Nature 322:445-448). Transgenes can be efficiently introduced into the ES cells by DNA transfection or by retrovirus-mediated transduction. Such transformed ES cells can thereafter be combined with blastocysts from a non-human animal. The ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal. For review see Jaenisch, R. (1988) Science 240:1468-1474.

Methods of making *HDx* knock-out or disruption transgenic animals are also generally known. See, for example, Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Recombinase dependent knockouts can also be generated, e.g. by homologous recombination to insert recombinase target sequences flanking portions of an endogenous *HDx* gene, such that tissue specific and/or temporal control of inactivation of an *HDx* allele can be controlled as above.

### *Exemplification*

The invention, now being generally described, will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention and are not intended to limit the invention.

#### Example 1

Trapoxin is a microbially derived cyclotetrapeptide that inhibits histone deacetylation *in vivo* and causes mammalian cells to arrest in the cell cycle. A trapoxin affinity matrix was used to isolate two nuclear proteins that copurified with histone deacetylase activity. Both proteins were identified by peptide microsequencing, and a cDNA encoding the histone deacetylase catalytic subunit (*HDI*) was cloned from a Jurkat T cell library. As the predicted protein is highly similar to the yeast transcriptional regulator RPD3, this study supports a role for histone deacetylase as a key regulator of eukaryotic transcription.

A requirement for a functional histone deacetylase in cell cycle progression has been implicated by the discovery that two cytostatic agents, trapoxin and trichostatin (Figure 1A), inhibit histone deacetylation in cultured mammalian cells and in fractionated cell extracts (4). In addition to causing G<sub>1</sub> and G<sub>2</sub> phase cell cycle arrest, these natural products alter gene expression and induce certain mammalian cell lines to differentiate. Whereas sodium butyrate also has these properties, both trapoxin and trichostatin are five orders of magnitude more potent.

Trapoxin is an "irreversible" inhibitor of histone deacetylase activity and its molecular structure offers clues as to how it could form a covalent bond with a nucleophilic active site residue. First, trapoxin contains an electrophilic epoxyketone that is essential for biological activity (5). Second, the aliphatic epoxyketone side chain is approximately isosteric with N-acetyl lysine (Figure 1A). Trapoxin likely acts as a substrate mimic, with epoxyketone poised to alkylate an active site nucleophile. We therefore regarded trapoxin as a tool that could reveal the molecular identity of histone deacetylase, so that its role in transcriptional regulation and cell cycle progression could be elucidated.

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Tritium-labeled trapoxin was prepared by total synthesis and used to identify trapoxin binding protein in crude extracts from bovine thymus. We used a charcoal precipitation assay to detect a specific trapoxin binding activity primarily in the nuclear fraction of the extracts (6). The binding activity was saturable with nanomolar concentrations of [<sup>3</sup>H]trapoxin and was completed by the simultaneous addition of unlabeled trapoxin. Trichostatin also competed with [<sup>3</sup>H]trapoxin (for synthesis, see Example 2), suggesting that both of these compounds exert their cellular effects by targeting the same molecule.

If trapoxin and trichostatin induce cell cycle arrest by directly inhibiting histone deacetylase, then the binding and enzymatic activities should copurify. To investigate this possibility, we fractioned nuclear thymus proteins by ammonium sulfate precipitation and Mono Q anion exchange chromatography.

Briefly, thymocytes (~12 g) prepared from fresh bovine thymus were homogenized in hypotonic lysis buffer [20 mM tris (pH 7.8), 20 mM NaCl, 1 mM EDTA, 10% glycerol, 1mM PMSF, 1mM benzamidine, 10 µg/ml each of pepstatin, aprotinin, and leupeptin] by mechanical disruption and the nuclei were isolated by centrifugation at 3000g. Nuclei were resuspended in lysis buffer and the proteins were extracted with 0.4 M ammonium sulfate. The viscous lysate was sonicated and clarified by centrifugation at 100,000g for one hour. Proteins were then precipitated with 90% saturated ammonium sulfate and recovered by centrifugation (100,000g, one hour). After through dialysis against Q buffer (25 mM tris pH 8, 10 mM NH<sub>4</sub> Cl, 0.25 mM EDTA, 10% glycerol), a portion of the nuclear proteins (~12 mg total protein) was loaded onto a HR 10/10 Mono Q column (Pharmacia). The column was washed with 25ml Q buffer and eluted with a 50 ml linear gradient of 10 to 500 mM NH<sub>4</sub> Cl. The column was further washed with 25 ml 500 mM NH<sub>4</sub> and 25 ml 1 M histone deacetylase activities or further purified with the K-trap affinity matrix. All procedures were done at 4°C.

Two peaks of histone deacetylase activity eluted from the Mono Q column between 250 and 350 mM NH<sub>4</sub>Cl (Figure 1B). Trapoxin binding activity, as revealed by the charcoal precipitation assay (40 nM [<sup>3</sup>H]trapoxin), precisely coeluted with the histone deacetylase peaks. Furthermore, all detectable histone deacetylase activity was abolished by treatment with either trapoxin or trichostatin (20 nM). Similar results were obtained with Mono Q fractioned nuclear extracts prepared from human Jurkat T cells.

To purify the histone deacetylase further, we synthesized an affinity matrix based on the trapoxin structure. Because trapoxin itself is not amenable to derivatization and



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the epoxyketone side chain is indispensable for activity, we chose to replace one of the phenylalanine residues of trapoxin's cyclic core with a lysine that could then be covalently linked to a solid support. This molecule, which we call K-trap, was prepared by a twenty step synthesis starting with commercially available (R)-proline and (S,S)- threitol  
5 acetone (Figure 2A) (see Example 3). Synthetic K-trap inhibited [<sup>3</sup>H]thymidine incorporation in MG-63 human osteosarcoma cells with a potency approximately one tenth that of trapoxin. *In vitro* histone deacetylase activity was also inhibited potently by this compound (complete inactivation at 20 nM) (8).

K-trap was deprotected with Pd(Ph<sub>3</sub>P)<sub>4</sub> and coupled to an activated agarose  
10 matrix (Figure 2A). Mono Q fractions containing nuclear proteins from bovine thymus were incubated with the K-trap affinity matrix and then tested for both trapoxin binding and histone deacetylase activity. Both activities were depleted (90%) by treatment with the K-trap matrix, yet a control matrix capped with ethanolamine had no effect on either activity (8). Bound polypeptides were eluted by boiling the matrix in 1% SDS buffer and  
15 separated by polyacrylamide gel electrophoresis. *In vitro* binding experiments with soluble [<sup>3</sup>H]trapoxin indicated that the radiolabel is released into solution following protein denaturation with SDS or guanidinium hydrochloride. Thus, trapoxin binding proteins were expected to elute from the affinity matrix with SDS.

The silver stained gel of the affinity matrix eluates revealed six major polypeptides  
20 with apparent molecular sizes between 45 and 50kD (Figure 2B). The interaction between bovine p46-p50 and the K-trap matrix appeared to be specific, because these proteins were not retained when the incubation was done in the presence of either trapoxin or trichostatin (Figure 2B), nor were they structurally unrelated histone deacetylase inhibitor, trichostatin, to prevent p46-p50 from binding to the K-trap matrix  
25 implies that one or more of these polypeptides constitute the biologically relevant protein target of both trapoxin and trichostatin. When the affinity purification was repeated with Jurkat nuclear extracts, only two major bands, p50 and p55, were observed by silver staining (Figure 2B). Recovery of human p50 and p55 was similarly abolished by trapoxin (Figure 2B) and trichostatin (8). Because the relative intensities of bovine p46-  
30 p49 vary with each protein preparation, we suspect that they are proteolytic fragments derived from the bovine equivalent of human p55. One of the bands (p50) is common to both human and bovine sources.

Large scale purification of the bovine proteins led to the resolution of two major bands of ~46 and ~50 kD in the final preparative electrophoresis step, both of which  
35 were submitted for microsequencing.

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To obtain enough trapoxin binding protein for microsequencing, nuclear ammonium sulfate pellets from 15 bovine thymuses were prepared as described above. Sedimented proteins were resuspended in and dialyzed against buffer A [20 mM bistris (pH 7.2), 20 mM NaCl, 10% glycerol] for 12 hours, and brought to pH 5.8 by dialyzing  
5 against bugger A (pH 5.8) for 30 minutes. After centrifugation, the dialysate (~650 mg protein) was loaded onto a Q Sepharose FF column (2.6 x 10 cm; Pharmacia) and the column was washed with 120 ml buffer A (pH 5.8). Proteins wee eluted with a 400 ml linear gradient of 20 to 600 mM NaCl in buffer A. Fractions (10 ml; each fraction contained 1 ml of 1 M tris pH 8 to neutralize the acidic buffer A) were assayed for  
10 trapoxin binding activity. Tween-20 was added to active fractions at a final concentration of 0.05%, and these fractions were incubated with K-trap affinity matrix for 16 hours (25  $\mu$ l per ml Q fraction). After washing the matrix three times with phosphate buffered saline, bound proteins were eluted by boiling in 40  $\mu$ l of SDS sample buffer per 25  $\mu$ l of matrix. SDS eluates were combined and the proteins resolved by  
15 SDS-PVDF membrane (Biorad). Staining with Ponceau S revealed two major bands (46 and 50 kD). The excised bands were proteolytically digested and the HPLC purified peptide fragments were sequenced at the Harvard Microchemistry Facility.

The bovine protein of larger molecular size (~50 kD) corresponds to a known protein, RbAp48 (11), that consists of seven WD repeat domains (12). Originally  
20 identified as a protein that binds to the retinoblastoma gene product (pRb), RbAp48 may constitute an adaptor subunit that targets the histone deacetylase to specific chromatin domains.

The ~46 kD bovine protein is highly related to the protein encoded by the yeast RPD3 gene, which has been implicated by several genetic screens as a transcriptional  
25 regulator, but whose biochemical function is unknown (13). Partial cDNA sequences for the human gene were identified in the expressed sequence tag database (dbEST) and were used to design polymerase chain reaction (PCR) primers. Briefly, after noting sequence similarity between peptides derived from the purified bovine trapoxin binding protein and yeast RPD3, we checked dbEST to see whether any partial sequences for the  
30 human homologue had been reported. Two ESTs (Genbank accession numbers: D31480 and F07807) were identified whose predicted translation products aligned with high sequence similarity to NH<sub>2</sub>- and COOH-terminal regions of *HDI*, respectively, PCR primers were designed based on these tags and a one kilobase PCR product was obtained from a Jurkat cDNA library (Stratagene). A <sup>32</sup>P labeled probe prepared by random  
35 priming was used to screen the Jurkat library, and ten positive clones were isolated. One

of the clones was fully sequenced and found to contain a putative full-length open reading frame (Figure 3A). The peptide sequences obtained from the purified bovine protein align with 100% identity to sequences deduced from this coding region (Figure 3A, boxed residues). We call this human protein *HD1* (for histone deacetylase), and its  
5 predicted size of 55 kD agrees well with the estimated size of p55 isolated from Jurkat nuclear extracts using the K-trap affinity matrix (Figure 2B). A dbEST search indicated the existence of at least two other related human genes.

To determine the relationship between the proteins from bovine thymus (p46-p50) and the proteins isolated from human Jurkat T cells (p50 and p55), an antiserum  
10 was generated against a peptide specified by the *HD1* open reading frame (Figure 3A, amino acids 319 to 334). Immunoblot analysis of the bovine proteins p46-p49 and the human protein p55 showed that they all react with the antiserum and provides additional evidence that these bands correspond to bovine and human *HD1* (Figure 3B). A monoclonal antibody that specifically recognizes RbAp48 was used to confirm the  
15 identity of bovine and hum p50. Importantly, neither *HD1* nor RbAp48 was detected when the affinity purification was done in the presence of trapoxin or trichostatin (Figure 3B).

We used affinity purified antibodies directed against a COOH-terminal peptide (amino acids 467 to 482) to immunoprecipitate *HD1* from crude nuclear extracts. The  
20 immunoprecipitates contained histone deacetylase activity that was inhibited by both trapoxin and trichostatin (Figure 4A). Consistent with the idea that *HD1* and RbAp48 form a complex *in vivo*, the two proteins coprecipitated with the anti-*HD1* antibodies (Figure 4B). Neither *HD1*, RbAp48, nor the associated histone deacetylase activity were immunoprecipitated in the presence of the *HD1* COOH-terminal peptide (Figure 4A and  
25 4B) (15). *HD1*, like RbAp48 (11), is detected predominantly in the nucleus by immunostaining with the aforementioned antibodies (8). Given that *HD1* and RbAp48 are the major proteins eluted from the K-trap matrix (Figure 2B), it is likely that they interact directly with one another.

We extended the results obtained with the endogenous protein by expressing  
30 recombinant FLAG epitope tagged *HD1* (*HD1*-F) in Jurkat T cells. Anti-FLAG immunoprecipitates from cells transfected with pBJ5/*HD1*-F contained histone deacetylase activity that was sensitive to both trapoxin and trichostatin (Figure 4C). Histone deacetylase activity was not precipitated when the antibody was blocked with excess FLAG peptide (15). Interestingly, endogenous RbAp48 did not coprecipitate with  
35 overexpressed *HD1*-F (8), demonstrating that RbAp48 is not required for either histone

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deacetylase or trapoxin binding activity. The result is consistent with the idea that RbAp48 serves a targeting rather than an enzymatic function. Finally, lysates from cells transfected with pBJ5/*HDI*-F were incubated with the K-trap affinity matrix in the presence or absence of trapoxin and trichostatin. Protein immunoblot analysis  
5 demonstrated an interaction between recombinant *HDI*-F and the K-trap affinity matrix that was fully competed by nanomolar concentrations of trapoxin or trichostatin (Figure 4D).

*HDI* is 60% identical to the protein encoded by the yeast *RPD3* gene, which was isolated in four independent mutant suppressor screens designed to identify  
10 transcriptional repressors (13, 16, 17, 18, 19). No biochemical function for the yeast protein has previously been postulated. A negative regulator of the *TRK2* gene, *RPD3* is necessary for the transcriptional repression of several genes whose expression is regulated according to specific environmental conditions. Loss of *RPD3* also leads to decreased transcriptional activation of certain genes, but this effect may be indirect (13,  
15 17). Although *RPD3* had yet to be implicated in silencing at telomeres or the mating loci, the fact that silencing is eliminated by point mutations in specific lysine residues near the  $\text{NH}_2$ -terminus of histones H3 and H4 suggests that lysine deacetylation may contribute to the maintenance of silenced chromatin (20, 21, 22, 23). Indeed, silencing at telomeres and the mating loci has been correlated with the presence of hypoacetylated  
20 histones, and sir mutants which are defective in silencing show a corresponding increase in the extent of histone acetylation at these loci (24). The *SIR3* and *SIR4* proteins have been shown to interact with a bacterially expressed histone H4  $\text{NH}_2$ -terminal domain *in vitro* (25), and it is possible that deacetylation of one or more lysine residues is required for this interaction *in vivo*. Our results further support a role for histone deacetylase as a  
25 transcriptional regulator and establish a biochemical connection to the genetic studies that originally characterized *RPD3*.

How does inhibition of histone deacetylase in mammalian cells lead to  $G_1$  and  $G_2$  phase cell cycle arrest? One possibility is that specific cell cycle regulatory proteins such as the cyclin dependent kinase inhibitors are transcriptionally upregulated in response to  
30 histone deacetylase inactivation. Alternatively, cell cycle checkpoints may exist that monitor histone acetylation or higher-order chromatin structure. It should now be possible to study the regulation of histone deacetylase during the cell cycle, its substrate specificity, and the mechanism by which it is targeted to specific regions of the genome.

Example 2

3H-Trapoxin was prepared from (S,S)-threitol acetonide (9) by total synthesis, as outlined in Figures 8A-8C.

As shown in Figure 8A, (S,S)-threitol acetonide (9) was monoprotected by treatment with triisopropylsilylchloride (TIPSCI) and sodium hydride in tetrahydrofuran (THF). The free alcohol was then subjected to Swern oxidation. Wittig reaction of the resulting aldehyde gave compound 10 in good yield for the three steps. Compound 10 was then hydrogenated with deprotection of the primary alcohol, which was then converted to the bromide 11 in excellent yield. Bromide 11 was converted to the organocuprate and reacted with (S)-serine  $\beta$ -lactone to yield the benzyloxycarbonyl-(Cbz) protected amino acid 12.

As shown in Figure 8B, 12 was coupled to tripeptide methyl ester 14, and the methyl ester was saponified. The amino acid was then cyclized and the silyl protecting group was removed to yield cyclotetrapeptide 18 in 51% yield.

Cyclotetrapeptide 18 was tritiated, as shown in Figure 8C, by oxidation of the primary alcohol with the Dess-Martin reagent, and the aldehyde was reduced with tritiated sodium borohydride to provide tritiated 18, which was converted to [ $^3\text{H}$ ]Trapoxin B by tosylation of the primary alcohol, deprotection of the diol, epoxide ring closure, and oxidation of the secondary alcohol to yield the desired compound. Non-radiolabelled 18 was converted to [ $^3\text{H}$ ]Trapoxin B, via tosylate 19, in 68% overall yield.

Example 2

K-Trap was prepared from (S,S)-threitol acetonide (9) by total synthesis, as outlined in Figures 9A-9C. As shown in Figure 9A, monoprotection and Swern oxidation of 9 yielded the aldehyde as above. Wittig homologation yielded carboxylic acid 20, which was converted to the mixed anhydride and treated with lithiated oxazolidinone 21 to provide 22 in excellent yield. Deprotection of the primary alcohol and conversion to the tosylate were followed by treatment of the potassium enolate with trisylazide according to the method of Evans to effect electrophilic azide transfer in good overall yield and stereoselectivity, providing compound 23. Removal of the chiral auxiliary and catalytic reduction of the azido function, with hydrogenation of the olefin, provided amino acid 24, which was N-protected to give the Fmoc derivative 25 in high overall yield.



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Referring to Figure 9B, protected amino acid 25 was coupled to tripeptide methyl ester 26. The methyl ester was saponified to yield 27, which was cyclized under high-dilution conditions to provide cyclotetrapeptide 28 in 58% yield..

As shown in Figure 9C, compound 28 was converted to K-trap (29) by  
5 deprotection of the diol, base-promoted epoxide closure, and oxidation of the secondary alcohol to provide K-trap (29) in good overall yield. The K-trap affinity matrix 30 was provided by palladium-catalyzed removal of the allyloxycarbonyl (Alloc) group from the lysine residue of 29, and immobilization on Affigel 10.

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### Example 3

#### Histone Deacetylase Activity is Required for Full Transcriptional Repression by mSin3A

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The Mad family of basic region-Helix-Loop-Helix-Leucine Zipper (BHLHZip) proteins play an important role in controlling cell proliferation and differentiation (for reviews see: Amati and Land, 1994; Bernards 1995). Four identified Mad family members: Mad1, Mx1, Mad3 and Mad4 (Ayer et al., 1993; Zervos et al., 1993; Hurlin et al., 1995a) form heterodimers with another BHLHZip protein, Max; to repress transcription (Ayer et al., 1993; Hurlin et al., 1995a) and are thought to play a negative role in the control of cell proliferation.

Two mammalian homologs of the *Saccharomyces cerevisiae* transcriptional corepressor SIN3, mSin3A and mSin3B, have recently been identified as Mad interacting proteins and are required for Mad-mediated transcriptional repression (Ayer et al., 1995; Schreiber-Agus et al., 1995). The most conserved regions of these proteins correspond to four putative paired amphipathic helix (PAH) motifs, which have been proposed to constitute protein-protein interaction surfaces (Wang et al., 1990). The second PAH motif in mSin3A, mSin3B and Sin3p interacts with the mSin3 interaction domain or SID in the amino terminus of the four Mad family members (Ayer et al., 1995; Schreiber-Agus et al., 1995; Hurlin et al., 1995a; Kasten et al., 1996). Mad1, Max and mSin3A form ternary complexes capable of binding DNA (Ayer et al., 1995). Point mutations in the SID domain of Mad1 disrupt its ability to bind mSin3A, negate its function as a transcriptional repressor (Ayer et al., 1995), and eliminate Mad1 function in several biological assays (Koskinen et al., 1995; Roussel et al., 1996). These findings suggest that Mad:Max heterocomplexes repress transcription by tethering either mSin3A or mSin3B to DNA. A chimeric protein fusing the SID of Mad1 to the GAL4 DNA-binding domain results in repression of simple and complex promoters in a manner that is dependent on mSin3 binding, suggesting that targeting mSin3 to DNA is necessary for

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repression (Ayer et al., 1996). Nevertheless, the molecular mechanism(s) for mSin3A-mediated repression remain unknown.

As described in example 1, a mammalian histone deacetylase has been identified, and cDNAs encoding the protein, histone deacetylase 1 (HD1 or HDAC1 for the purposes of this example), have been cloned (see also Taunton et al., 1996b). HDAC1 is approximately 60% identical to the *S. cerevisiae* RPD3 protein, which is a component of a yeast histone deacetylase complex (Rundlett et al., 1996). Single mutations in either RPD3 or SIN3 give the same phenotypes as RPD3/SIN3 double mutants suggesting that they function in the same pathway (Stillman et al., 1994). Because Mad family proteins use mSin3A as a corepressor and Mad1 can repress transcription in wild-type yeast but not yeast having a null mutation in SIN3 (Kasten et al., 1996) or RPD3 (D.J. Stillman, personal comm.), it is likely that the mechanism of transcriptional repression by Mad proteins may be conserved between yeast and higher eukaryotes. Consistent with this hypothesis, the DNA-binding transcription factor YY1 interacts with a mammalian RPD3 homolog to repress transcription of a heterologous reporter gene (Yang et al., 1996). These results demonstrate that mammalian RPD3-like activity functions in transcriptional regulation.

Several lines of evidence suggest that the acetylation status of conserved lysines in the amino terminal domains of histones H3 and H4 play a role in the regulation of transcription. In general, histone hyperacetylation correlates with transcriptionally active or poised genes; conversely, hypoacetylation correlates with transcriptionally repressed heterochromatin (for reviews see: Turner, 1993; Loidl, 1994; Wolffe, 1996). While little is known about the targeting and regulation of histone acetyltransferases and deacetylases, it has been recently shown that several transcriptional coactivators possess inherent acetyltransferase activity (Brownell et al., 1996; Ogryzko et al., 1996) or associate with acetyltransferases (Yang et al., 1996b). We report that mSin3A and HDAC1 associate in vivo and that the histone deacetylase inhibitor trapoxin interferes with mSin3A-mediated transcriptional repression.

## 30 Results

(i) mSin3A is present in cells as a large stable multiprotein complex.

To study the *in vivo* function of mSin3A we generated polyclonal antiserum specific for the PAH2 domain of mSin3A. We tested this antiserum by immunoprecipitation using nuclear lysates made from the myeloid leukemia cell line U937

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that had been metabolically labeled with <sup>35</sup>S-methionine. Analysis of immunoprecipitates showed an intensely-labeled doublet with an apparent molecular weight of 150 kiloDaltons that was present in the anti-mSin3A immunoprecipitates (Figure 10A). This doublet comigrated with *in vitro* translated-mSin3A, shared identical V8 protease digestion peptides with *in vitro* translated-Sin3A and was absent from immunoprecipitations using preimmune serum or immune serum preincubated with the cognate immunogen (data not shown).

Fractionation of U937 nuclear extracts by size exclusion chromatography indicated that mSin3A is present in large molecular weight complex(es) (D.E.A., unpublished). To address this possibility, we performed immunoprecipitations from metabolically-labeled U937 cells under conditions that should preserve protein-protein interactions. In addition to mSin3A, the low-stringency mSin3A immunoprecipitates contained several labeled polypeptides of apparent molecular weight 250 kDa, 180 kDa, 55 kDa, 50 kDa, 42 kDa, 33-36 kDa and 30 kDa (Figure 10A). These proteins were not detected in immunoprecipitates using mSin3A antiserum blocked with the cognate immunogen, suggesting that the proteins detected are specifically associated with mSin3A. Furthermore, none of these proteins were detected using high-stringency immunoprecipitation or by western blotting of whole-cell lysates using anti-Sin3A, suggesting that they do not share epitopes with mSin3A and are not proteolytic breakdown products of mSin3A (data not shown). All of the associated proteins appear to be present in substoichiometric amounts to mSin3A, suggesting that mSin3A complexes are heterogeneous.

To test the stability of the mSin3A complex, we subjected low-stringency mSin3A immunoprecipitates to different salt concentrations and ionic detergent conditions. The proteins that remained bound to mSin3A in the immune complex were analyzed by SDS-PAGE. Under the most stringent conditions we observed only a slight loss of mSin3A-associated proteins in the immune complex (Figure 10B). One exception to this finding was the apparently quantitative loss of p42 under slightly-elevated salt concentrations. These findings demonstrate that the mSin3A complex is stable *in vivo* and suggests that some or all of the mSin3A-associated proteins may facilitate mSin3A function as a transcriptional co-repressor.

(ii) HDAC1 and RbAp48 are components of the mSin3A complex.

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Because SIN3 and RPD3 appear to function in the same pathway in yeast and two components of the mSin3A complex, p50 and p55, are similar in apparent molecular weight to HDAC1 we hypothesized that HDAC1 or related proteins might be components of the mSin3A repressor complex. To test this hypothesis, the proteins  
5 bound to mSin3A immunocomplexes were eluted with ionic detergents and reprecipitated with affinity purified antibodies specific for an internal peptide of HDAC1. Only two proteins eluted from the mSin3A complex were re-precipitated by HDAC1 antiserum (Figure 11A). These polypeptides comigrated with p50 and p55 from the low stringency mSin3A immunoprecipitation, suggesting that proteins highly related to HDAC1 are  
10 complexed to mSin3A *in vivo*. p55 comigrates with *in vitro* translated HDAC1 and is recognized by an antibody specific for a carboxy-terminal epitope unique to HDAC1. Another CDNA encoding an HDAC1 homolog, HDAC2, has recently been identified (Yang et al., 1996a). It is likely that p50 represents HDAC2 (data not shown).

In a reciprocal experiment, we performed low stringency immunoprecipitations  
15 using antiserum specific for an epitope at the carboxy-terminus of HDAC1. HDAC1 immunoprecipitates contain several proteins that were specifically competed with the immunizing peptide (Figure 11B). A polypeptide doublet that comigrated with mSin3A was detected in the HDAC1 immunocomplexes (Figure 11B and 11C). To confirm that the doublet coprecipitating with HDAC1 is mSin3A, the HDAC1 immunocomplex was  
20 eluted and reprecipitated with antiserum specific for mSin3A (Figure 11C). The two proteins in this precipitate comigrated with mSin3A, confirming that mSin3A and HDAC1 are associated *in vivo*.

To determine whether HDAC1 associated with mSin3A *in vivo* is enzymatically active, we assayed low-stringency immunoprecipitates for histone deacetylase activity.  
25 We used a synthetic peptide corresponding to the first twenty four amino acids of histone H4 as a substrate for our deacetylase assay (Taunton et al., 1996b). Low-stringency anti-mSin3A immunoprecipitates contained deacetylase activity; however, only background levels of deacetylase activity were detected in the immunoprecipitates if the mSin3A antiserum was blocked with cognate immunogen (Figure 11D). To confirm the authenticity  
30 of the mSin3A associated activity we treated the immunoprecipitates with synthetic trapoxin, a specific inhibitor of histone deacetylase activity (Taunton et al., 1996a). Treatment of mSin3A complexes *in vitro* with 10 nM trapoxin reduced deacetylation by approximately 50% (Figure 11D), suggesting that the precipitated deacetylase activity can be attributed to trapoxin-sensitive histone deacetylases bound to mSin3A.



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We detected an interaction between HDAC1 and RbAp48 *in vivo* (Figure 11B, 11C and Example 1). The low-stringency mSin3A immunoprecipitation shown in Figure 11C also contained a protein that comigrated with RbAp48 (marked with an asterisk) that was not readily visible on the shorter exposures of low stringency mSin3A immunoprecipitations (Figure 11A). We have identified RbAp48 in mSin3A immunoprecipitates from cell extracts of nontransfected cells by western blotting, further demonstrating that mSin3A and RbAp48 associate *in vivo* (Figure 12A).

To address further the association between HDAC1 and RbAp48 with mSin3A, we expressed the mammalian proteins in insect cells using recombinant baculoviruses. To this end, we expressed recombinant FLAG-epitope tagged HDAC1 (HDAC1-F) that could be immuno-purified by anti-FLAG antibodies and histidine-tagged mSin3A (mSin3A-H) that could be purified by nickel affinity (data not shown). HDAC1-F was immunoprecipitated from infected Sf9 cell extracts by anti-FLAG antibodies in the presence or absence of mSin3A-H. HDAC1-F was also precipitated by Ni<sup>2+</sup>-NTA agarose in a manner that was dependent on coexpression of mSin3A-H (Figure 12B), demonstrating that a complex between HDAC 1 and mSin3A is formed in insect cells using exogenously expressed human proteins.

Consistent with our finding that RbAp48 is associated with mSin3A and HDAC1 *in vivo*, we show that baculovirus expressed Flu-epitope tagged RbAp48 (p48-HA) is specifically precipitated from infected Sf9 cell extracts using anti-FLAG antibody only when HDAC1-F is coexpressed. Furthermore, p48-HA is specifically retained by Ni<sup>2+</sup>-NTA in the presence of mSin3A-H (Figure 12C). Co-expression of p48-HA did not appear to effect the association between HDAC1-F and mSin3A-H, suggesting that the regions of interaction are distinct and that all three proteins can associate simultaneously. These data suggest a direct interaction between mSin3A, HDAC1 and RbAp48 *in vivo*.

(iii) Transcription repression by mSin3A requires histone deacetylase activity.

To investigate whether histone deacetylation plays a role in mSin3A-mediated transcriptional repression *in vivo*, we examined mSin3A-specific repression in the presence and absence of the histone deacetylase inhibitor trapoxin. 293 cells were transfected with a luciferase reporter gene construct containing a minimal promoter consisting of only a TATA box and initiation site derived from the myelom nocyctic growth factor gene (Figure 13A). This reporter has four consensus binding sites for the DNA binding domain of the *S. cerevisiae* transcriptional activator GAL4 and therefore is

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responsive to chimeric proteins containing the GAL4 DNA binding domain (GALDBD) (Sterneck et al., 1992). We have used this reporter construct previously to demonstrate that fusion of the SID repressor region of Mad1 to the GALDBD is necessary for mSin3A-dependent transcriptional repression. Furthermore, we have shown that fusion  
5 of SID to the potent transcriptional activator GALVP16, MadN35GALVP16, can cancel the activation function of VPI6 in an mSin3A-dependent manner. Consistent with our previous results (Ayer et al., 1996), MadN35GALVP16 activated transcription from the reporter gene approximately 100-fold less well than GALVP16 (data not shown). As a negative control we engineered two proline substitutions into the SID of Mad1,  
10 Mad(Pro); this protein cannot bind mSin3A *in vitro* (Ayer et al., 1995). Consistent with an inability to interact with mSin3A, Mad(Pro)GALVP16 is a much less potent repressor (Figure 13B). In control experiments we have shown that the observed effects require the presence of GAL4 sites in the promoter and that both MadN35GALVP16 and Mad(Pro)N35GALVP16 are expressed to equivalent levels in these cells and bind GAL4  
15 sites with similar affinities (data not shown). To test the role of histone deacetylation on the repression observed in our transfection assays, we first examined the effect of trapoxin on histone deacetylase activity in 293 cells. As expected, *in vivo* treatment with 10 nM trapoxin for eight hours reduced deacetylase activity of both crude 293 extracts and anti-HDAC1 immunopurified complexes by approximately 46% and 58%,  
20 respectively (Figure 13C).

To test the effect of a histone deacetylase inhibitor on MadN35GALVP16 and Mad(Pro)N35GALVP16 mediated repression, we treated a duplicate set of transfections with 10 nM trapoxin for eight hours prior to harvest. In the representative experiment shown, 10 nM trapoxin treatment derepressed the activity of MadN35GALVP16 nine-  
25 fold while it had little effect on the activity of Mad(Pro)N35GALVP16, suggesting that the histone deacetylation plays a direct role in mSin3A transcriptional repression (Figure 13B). In addition, there was typically less than a two-fold effect of trapoxin on the activity of the reporter gene in cells transfected with the expression vector alone or in cells transfected with GALVP16 (data not shown). Following trapoxin treatment, the  
30 repression observed for MadN35GALVP16 was still seven times greater than that of Mad(Pro)N35GALVP16, suggesting that the residual deacetylase activity following trapoxin treatment (Figure 13B) continues to drive mSin3A-mediated repression; however, we can not rule out that mSin3A is capable of repression by mechanisms independent of histone deacetylation.

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### Discussion

Earlier studies implicated mSin3 as the primary candidate for the negative transcriptional function of the DNA binding transcription factor Mad (Ayer et al., 1995; Schreiber-Agus et al. 1995; Ayer et al., 1996). We present evidence that the mSin3A compressor is part of a high molecular weight, multicomponent complex(es) that contains active histone deacetylase, thereby implicating histone deacetylation as a potential mechanism for mSin3A-mediated repression. Furthermore, we observe a pronounced increase in the transcriptional activity of an mSin3A-silenced reporter gene upon treatment *in vivo* with the specific histone deacetylase inhibitor trapoxin, suggesting that full transcriptional repression by mSin3A requires histone deacetylation. These results suggest a mechanism of gene regulation through the targeting of an enzyme that alters chromatin structure.

These observations are consistent with genetic experiments in yeast, suggesting that the yeast orthologs of mSin3A and HDAC1, SIN3 and RPD3 respectively, are epistatic transcriptional regulators (Stillman et al., 1994). Furthermore, recent biochemical evidence demonstrates that Rpd3p is a component of a large molecular weight histone deacetylase complex in yeast (Rundlett et al., 1996). Together with our results, these findings predict a conservation of the mSin3/HDAC1 functional association in yeast.

We have used chimeric transcriptional regulators to discern the effects of trapoxin on the activity of our reporter genes. The MadN35GALVP16 chimera functioned as a repressor by a mechanism that was dependent on the binding of mSin3A and that was sensitive to trapoxin. The same mutations that inactivate MadN35GALVP16 as a transcriptional repressor (i.e. Mad(Pro)N35GALVP16), also block interaction between Mad1 and mSin3A *in vitro* and Mad1 function *in vivo*. Therefore, it is likely that Mad:Max heterocomplexes repress transcription in a manner dependent on an mSin3A-associated histone deacetylase.

By co-immunoprecipitation we have demonstrated that mSin3A and HDAC1 associate *in vivo*. Consistent with these data we observed nuclear colocalization of mSin3A and HDAC1 by immunofluorescence microscopy (data not shown). Finally, overexpression in insect cells facilitates co-purification of mSin3A and HDAC1 (Figure 12B and C), suggesting that the interaction between mSin3A and HDAC1 is either direct or requires a conserved cofactor. The finding that mSin3A has different associated histone deacetylases (HDAC1 and HDAC2) suggests that the mSin3A complex(es) may

have multiple substrate or target specificities. The heterogeneous nature of the mSin3A complex potentially reflects a diverse array of repressors, histone deacetylases and different targeting molecules that facilitate mSin3A-dependent alterations in gene expression.

5       At least five additional polypeptides are stably associated with mSin3A (Figures 10 and 11); whose function is currently unknown but tight association with mSin3A in both U937 cells and Jurkat T cells (data not shown) suggests that they in some way mediate mSin3A function. Furthermore, we have identified an association between mSin3A and RbAp48 *in vivo*, suggesting that this protein may play a role in regulating  
10 mSin3A-targeted deacetylation. RbAp48 was originally identified as a retinoblastoma binding protein that contains WD repeats and shares homology with the  $\gamma$ -subunit of G-proteins (Qian et al., 1993). Subsequently, it has been shown that RbAp48 or its orthologs are involved in targeting different histone modifying enzymes to chromatin (Parthun et al., 1996; Taunton et al., 1996b; Tyler et al., 1996; Verreault et al., 1996).  
15 The mSin3A/RbAp48 complex isolated from U937 cells (Figure 11) is likely to represent only a small fraction of the mSin3A complexes, but its detection implies that mSin3A may play a role in the control of different aspects of chromatin physiology as well as transcription repression.

It is unclear how different chromatin states facilitate transcription repression and  
20 activation or how their distinct biochemical states arise; however, there is ample cytological, genetic and biochemical evidence supporting the model that hyperacetylated chromatin is transcriptionally more active than hypoacetylated chromatin. Acetylation levels in  $\gamma$ -heterochromatin of *Drosophila melanogaster* polytene chromosomes are significantly reduced at lysine positions 5, 8, and 16 of histone H4, while the  
25 transcriptionally hyperactive X-chromosome of male flies is uniquely hyperacetylated at position 16 (Turner et al., 1992). In yeast, mutation of acetyl-accepting lysines in histone H4 reduces the activity of the *GALI*, *PHOS* and *CUP1* promoters *in vivo* (Durrin et al., 1991). The transcriptionally silent regions in yeast, HML and HMR, are hypoacetylated and their activation is correlated with acetylation of histone H4 (Braunstein et al., 1993).  
30 Additionally, biochemical studies showed that certain transcription factors have higher affinity for their binding sites when those sites are embedded in chromatin assembled from hyperacetylated histones (Lee et al., 1993; Vettes-Dadey et al., 1996). Finally, evidence suggesting that acetylation is required for activation comes from the recent demonstration that several coactivators either encode acetyltransferases or are associated with  
35 acetyltransferases. Thus, our data support this general model for the control of gene

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expression by histone acetylation status and provide a biochemical mechanism for deacetylation-mediated repression.

The acetylation status of a particular chromatin region represents a balance between competing, acetylation and deacetylation reactions. We propose that  
5 MadN35GALVP16 recruits mSin3A-HDAC complexes to specific sites on DNA and shifts this equilibrium towards deacetylation and subsequent transcription repression by creating a high effective molarity of the histone deacetylase. In yeast, the activation domain of VP16 has been shown to use the acetyltransferase Gen5p as a coactivator (Marcus et al., 1994; Brownell et al., 1996), suggesting that in mammalian cells VP16  
10 will also use an acetyltransferase as a cofactor. Thus, trapoxin treatment could shift the equilibrium from deacetylation to acetylation and thereby drive activation.

Whether histone deacetylation will always have a negative effect on gene expression is unclear. Mutants in SIN3 and RPD3 can have both positive and negative effects on gene expression (Vidal and Gaber, 1991; Yoshimoto et al., 1992); however,  
15 for SIN3 there is evidence that positive effects may be indirect (Wang et al., 1994). In addition, mutations or deletions in RPD3 have recently been shown to enhance telomeric silencing both in yeast and in fruit fly (Sussel et al., 1995; De Rubertis et al., 1996; Rundlert et al., 1996). In mammalian cells, deacetylase inhibitors can inhibit MyoD- (Johnston et al., 1992) and steroid receptor-activated transcription (McKnight et al.,  
20 1990; Bresnick et al., 1990). While it remains to be shown that the effects of RPD3 on silencing are direct, this evidence suggests that histone deacetylation can elicit both positive and negative effects on gene expression. Determining the factors that govern the functional outcome of histone deacetylation will provide fertile ground for further experimentation.

25

### *Experimental Procedures*

Antibodies, cell culture, and Immunoprecipitations: To generate antiserum specific for mSin3A a GST fusion protein encoding, amino acids 251 through 405 of mSin3A was used to immunize a New Zealand White rabbit. The crude serum was  
30 passed over a GST column to remove the anti-GST antibodies. U937 cells were grown in RPMI supplemented with 10% calf serum (Hyclone), glutamine and penicillin-streptomycin. Low and high stringency immunoprecipitations were performed essentially as described (Ayer and Eisenman, 1993). To elute proteins from low stringency



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immunoprecipitates, they were incubated for 60 minutes at room temperature in antibody buffer and reprecipitated under high stringency conditions.

Luciferase assays: 293 cells were seeded in triplicate onto 60 mm dishes at  $3 \times 10^5$  cells in 4 ml DME with 10% calf serum (Hyclone). Six hours after seeding, cells were transfected with 50 ng luciferase reporter, 50 ng CMV- $\beta$ -gal, 50 ng expression construct, and 2.85  $\mu$ g carrier DNA using the BBS/CaPO<sub>4</sub> method. 10 nM trapoxin was added to the media 8 hours prior to the luciferase assays. Cell lysates were prepared 20-24 hours following transfections, and luciferase and  $\beta$ -galactosidase activities was assayed according to manufacturer directions (Promega, Tropix). Luciferase values (relative light units) were normalized for transfection efficiency by dividing by  $\beta$ -gal activity.

Histone deacetylase assays: *In vitro* histone deacetylase activity was assayed essentially as described with either 50  $\mu$ l of crude cell extract (approximately  $5 \times 10^6$  Cells) or immunopurified cell extracts (approximately  $2 \times 10^7$  cells) for 2.5 hrs at 37 C (Taunton et al., 1996b). Pretreatment of crude or immunopurified extract with synthetic trapoxin was performed for 30 minutes at 4 C prior to addition of peptide substrate. TAG Jurkat and 293 cell extracts for histone deacetylase assays were prepared as in Taunton et al., 1996. Anti-HDAC1 and anti-mSin3A immunoprecipitations were performed as described above and in Figure 11. The Protein-A conjugated immunoprecipitates were washed three times in J-buffer plus 1 mM EDTA and resuspended in J-buffer without Triton-X-100, and histone deacetylase activity was measured as described.

Baculoviruses: cDNAs encoding Flag-tagged HDAC1, HA-tagged RbAp48 and His-tagged mSin3A were cloned into the transfer vector pVL 1392 (specific details on the construction of these vectors is available upon request). Recombinant virus was generated using Baculogold DNA according to the manufactures instructions (Pharminigen). Sf9 or High 5 cells were infected at high multiplicity, extracts prepared 48 hours post infection and immunoprecipitations performed as described above. Ni<sup>2+</sup>-NTA agarose and anti-Flag antibody were purchased from Qiagen and Kodak-IBI, respectively.

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- Wang, H., Reynolds-Hager, L., and Stillman, D. J. (1994). Genetic interactions between SIN3 mutations and the *Saccharomyces cerevisiae* transcriptional activators encoded by MCM1, STE12, and SWII. *Mol. Gen. Genet.* 245, 675-685.
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- 40 Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (1996b)- A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* 382, 319-324.
- Yoshimoto, H., Ohmae, M., and Yamashita, I. (1992). The *Saccharomyces cerevisiae* GAM2/SIN3 protein plays a role in both activation and repression of transcription. *Mol.*  
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5

#### *Example 4*

Since the priority date of this application, a number of other mammalian HDx genes have been described in the literature. In particular, a mouse HD1 clone is identified in GenBank as accession number U807080. Another HDx member, HD2 (HDAC-2) is also described for both human and mouse; see for example, GenBank  
10 entries U31814 and U31758. Without exception, each clone includes a  $\nu$  motif represented in the general formula of SEQ ID No. 12, and a  $\chi$  motif represented in the general formula SEQ ID No. 14.

All of the above-cited references and publications are hereby incorporated by  
15 reference.

#### *Equivalents*

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific polypeptides, nucleic acids, methods, assays and reagents described herein. Such equivalents are considered to be  
20 within the scope of this invention and are covered by the following claims.



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## SEQUENCE LISTING

- 5 (1) GENERAL INFORMATION:
- (i) APPLICANT: Schreiber, Stuart L.  
Taunton, Jack  
Hassig, Christian A.  
Jamison, Timothy F.
- 10 (ii) TITLE OF INVENTION: Histone Deacetylases and Uses Related Thereto
- (iii) NUMBER OF SEQUENCES: 15
- 15 (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: FOLEY, HOAG & ELIOT, LLP  
(B) STREET: One Post Office Square  
(C) CITY: Boston  
20 (D) STATE: MA  
(E) COUNTRY: USA  
(F) ZIP: 02109
- (v) COMPUTER READABLE FORM:
- 25 (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: ASCII(text)
- 30 (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER: US  
(B) FILING DATE: 26-MAR-1996  
(C) CLASSIFICATION:
- 35 (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Vincent, Matthew P.  
(B) REGISTRATION NUMBER: 36,709  
(C) REFERENCE/DOCKET NUMBER: HUV019.25
- 40 (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: (617) 832-1000  
(B) TELEFAX: (617) 832-7000
- 45 (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1449 base pairs  
(B) TYPE: nucleic acid  
50 (C) STRANDEDNESS: b th  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA

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## (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..1446

5

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

10	ATG GCG CAG ACG CAG GGC ACC CGG AGG AAA GTC TGT TAC TAC TAC GAC	48
	Met Ala Gln Thr Gln Gly Thr Arg Arg Lys Val Cys Tyr Tyr Tyr Asp	
	1 5 10 15	
15	GGG GAT GTT GGA AAT TAC TAT TAT GGA CAA GGC CAC CCA ATG AAG CCT	96
	Gly Asp Val Gly Asn Tyr Tyr Tyr Gly Gln Gly His Pro Met Lys Pro	
	20 25 30	
20	CAC CGA ATC CGC ATG ACT CAT AAT TTG CTG CTC AAC TAT GGT CTC TAC	144
	His Arg Ile Arg Met Thr His Asn Leu Leu Leu Asn Tyr Gly Leu Tyr	
	35 40 45	
25	CGA AAA ATG GAA ATC TAT CGC CCT CAC AAA GCC AAT GCT GAG GAG ATG	192
	Arg Lys Met Glu Ile Tyr Arg Pro His Lys Ala Asn Ala Glu Glu Met	
	50 55 60	
30	ACC AAG TAC CAC AGC GAT GAC TAC ATT AAA TTC TTG CGC TCC ATC CGT	240
	Thr Lys Tyr His Ser Asp Asp Tyr Ile Lys Phe Leu Arg Ser Ile Arg	
	65 70 75 80	
35	CCA GAT AAC ATG TCG GAG TAC AGC AAG CAG ATG CAG AGA TTC AAC GTT	288
	Pro Asp Asn Met Ser Glu Tyr Ser Lys Gln Met Gln Arg Phe Asn Val	
	85 90 95	
40	GGT GAG GAC TGT CCA GTA TTC GAT GGC CTG TTT GAG TTC TGT CAG TTG	336
	Gly Glu Asp Cys Pro Val Phe Asp Gly Leu Phe Glu Phe Cys Gln Leu	
	100 105 110	
45	TCT ACT GGT GGT TCT GTG GCA AGT GCT GTG AAA CTT AAT AAG CAG CAG	384
	Ser Thr Gly Gly Ser Val Ala Ser Ala Val Lys Leu Asn Lys Gln Gln	
	115 120 125	
50	ACG GAC ATC GCT GTG AAT TGG GCT GGG GGG CTG CAC CAT GCA AAG AAG	432
	Thr Asp Ile Ala Val Asn Trp Ala Gly Gly Leu His His Ala Lys Lys	
	130 135 140	
55	TCC GAG GCA TCT GGC TTC TGT TAC GTC AAT GAT ATC GTC TTG GCC ATC	480
	Ser Glu Ala Ser Gly Phe Cys Tyr Val Asn Asp Ile Val Leu Ala Ile	
	145 150 155 160	
60	CTG GAA CTG CTA AAG TAT CAC CAG AGG GTG CTG TAC ATT GAC ATT GAT	528
	Leu Glu Leu Leu Lys Tyr His Gln Arg Val Leu Tyr Ile Asp Ile Asp	
	165 170 175	
65	ATT CAC CAT GGT GAC GGC GTG GAA GAG GCC TTC TAC ACC ACG GAC CGG	576
	Ile His His Gly Asp Gly Val Glu Glu Ala Phe Tyr Thr Thr Asp Arg	
	180 185 190	

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	GTC ATG ACT GTG TCC TTT CAT AAG TAT GGA GAG TAC TTC CCA GGA ACT	624
	Val Met Thr Val Ser Phe His Lys Tyr Gly Glu Tyr Phe Pro Gly Thr	
	195 200 205	
5	GGG GAC CTA CGG GAT ATC GGG GCT GGC AAA GGC AAG TAT TAT GCT GTT	672
	Gly Asp Leu Arg Asp Ile Gly Ala Gly Lys Gly Lys Tyr Tyr Ala Val	
	210 215 220	
10	AAC TAC CCG CTC CGA GAC GGG ATT GAT GAC GAG TCC TAT GAG GCC ATT	720
	Asn Tyr Pro Leu Arg Asp Gly Ile Asp Asp Glu Ser Tyr Glu Ala Ile	
	225 230 235 240	
15	TTC AAG CCG GTC ATG TCC AAA GTA ATG GAG ATG TTC CAG CCT AGT GCG	768
	Phe Lys Pro Val Met Ser Lys Val Met Glu Met Phe Gln Pro Ser Ala	
	245 250 255	
20	GTG GTC TTA CAG TGT GGC TCA GAC TCC CTA TCT GGG GAT CGG TTA GGT	816
	Val Val Leu Gln Cys Gly Ser Asp Ser Leu Ser Gly Asp Arg Leu Gly	
	260 265 270	
	TGC TTC AAT CTA ACT ATC AAA GGA CAC GCC AAG TGT GTG GAA TTT GTC	864
	Cys Phe Asn Leu Thr Ile Lys Gly His Ala Lys Cys Val Glu Phe Val	
	275 280 285	
25	AAG AGC TTT AAC CTG CCT ATG CTG ATG CTG GGA GGC GGT GGT TAC ACC	912
	Lys Ser Phe Asn Leu Pro Met Leu Met Leu Gly Gly Gly Gly Tyr Thr	
	290 295 300	
30	ATT CGT AAC GTT GCC CGG TGC TGG ACA TAT GAG ACA GCT GTG GCC CTG	960
	Ile Arg Asn Val Ala Arg Cys Trp Thr Tyr Glu Thr Ala Val Ala Leu	
	305 310 315 320	
35	GAT ACG GAG ATC CCT AAT GAG CTT CCA TAC AAT GAC TAC TTT GAA TAC	1008
	Asp Thr Glu Ile Pro Asn Glu Leu Pro Tyr Asn Asp Tyr Phe Glu Tyr	
	325 330 335	
40	TTT GGA CCA GAT TTC AAG CTC CAC ATC AGT CCT TCC AAT ATG ACT AAC	1056
	Phe Gly Pro Asp Phe Lys Leu His Ile Ser Pro Ser Asn Met Thr Asn	
	340 345 350	
	CAG AAC ACG AAT GAG TAC CTG GAG AAG ATC AAA CAG CGA CTG TTT GAG	1104
	Gln Asn Thr Asn Glu Tyr Leu Glu Lys Ile Lys Gln Arg Leu Phe Glu	
	355 360 365	
45	AAC CTT AGA ATG CTG CCG CAC GCA CCT GGG GTC CAA ATG CAG GCG ATT	1152
	Asn Leu Arg Met Leu Pro His Ala Pro Gly Val Gln Met Gln Ala Ile	
	370 375 380	
50	CCT GAG GAC GCC ATC CCT GAG GAG AGT GGC GAT GAG GAC GAA GAC GAC	1200
	Pro Glu Asp Ala Ile Pro Glu Glu Ser Gly Asp Glu Asp Glu Asp Asp	
	385 390 395 400	
55	CCT GAC AAG CGC ATC TCG ATC TGC TCC TCT GAC AAA CGA ATT GCC TGT	1248
	Pro Asp Lys Arg Ile Ser Il Cys Ser Ser Asp Lys Arg Ile Ala Cys	

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	405	410	415	
5	GAG GAA GAG TTC TCC GAT TCT GAA GAG GAG GGA GAG GGG GGC CGC AAG Glu Glu Glu Phe Ser Asp Ser Glu Glu Glu Gly Glu Gly Gly Arg Lys 420 425 430			1296
10	AAC TCT TCC AAC TTC AAA AAA GCC AAG AGA GTC AAA ACA GAG GAT GAA Asn Ser Ser Asn Phe Lys Lys Ala Lys Arg Val Lys Thr Glu Asp Glu 435 440 445			1344
15	AAA GAG AAA GAC CCA GAG GAG AAG AAA GAA GTC ACC GAA GAG GAG AAA Lys Glu Lys Asp Pro Glu Glu Lys Lys Glu Val Thr Glu Glu Glu Lys 450 455 460			1392
20	ACC AAG GAG GAG AAG CCA GAA GCC AAA GGG GTC AAG GAG GAG GTC AAG Thr Lys Glu Glu Lys Pro Glu Ala Lys Gly Val Lys Glu Glu Val Lys 465 470 475 480			1440
25	TTG GCC TGA Leu Ala			1449

25 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 379 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

40	ATTGACTTCC TGCAGAGAGT CAGCCCCACC AATATGCAAG GCTTCACCAA GAGTCTTAAT	60
45	GCCTTCAACG TAGGCGATGA CTGCCCAGTG TTTCCCGGGC TCTTTGAGTT CTGCTCGCGT	120
	TACACAGGCG CATCTCTGCA AGGAGCAACC CAGCTGAACA ACAAGATCTG TGATATTGCC	180
50	ATTAAGTGGG CTGGTGGTCT GCACCATGCC TAGAAGTTTG AGGCCTCTGG CTTCTGCTAT	240
	GTCAACGACA TTGTGTTTGG CATCCTGGAG CTGCTCAAGT ACCACCCTCG GGTGCTCTAC	300
	ATTGACATTG ACATCCACCA TGGTGACGGG GTTCAAGAAG CTTTCTACCT CACTGACCGG	360
55	GTCATGACGG TGTCTTTC	379

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- 55 (A) LENGTH: 375 base pairs

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(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TACTACTGTC TGAACGTGCC CCTGCGGATG GGCATTGATG ACCAGAGTTA CAAGCACCTT 60  
TTCCAGCCGG TTATCAACCA GGTAGTGGAC TTCTACCAAC CCACGTGCAT TGTGCTCCAG 120  
15 TGTGGAGCTG ACTCTCTGGG CTGTGATCGA TTGGGCTGCT TTAACCTCAG CATCCGAGGG 180  
CATGGGGAAT GCGTTGAATA TGTCAAGAGC TTCAATATCC CTCTACTCGT GCTGGGTGGT 240  
GGTGGTTATA CTGTCCGAAA TGTGCCCCGC TGCTGGACAT ATGAGACATC GCTGCTGGTA 300  
20 GAAGAGGCCA TTAGTGAGGA GCTTCCCTAT AGTGAATACT TCGAGTACTT TGCCCCAGAC 360  
TTCACACTTC ATCCA 375

25 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 227 base pairs  
(B) TYPE: nucleic acid  
30 (C) STRANDEDNESS: both  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGTCATGCTA AATGTGTAGA AGTTGTAAAA ACTTTTAACT TACCATTACT GATGCTTGGA 60  
40 GGAGGTGGCT ACACAATCCG TAATGTTGCT CGATGTTGGA CATATGAGAC TGCAGTTGCC 120  
CTTGATTGTG AGATTCCCAA TGAGTTGCCA TATAATGATT ACTTTGAGTA TTTTGGACCA 180  
45 GACTTCAAAC TGCATATTAG TCCTTCAAAC ATGACAAACC AGAACAC 227

(2) INFORMATION FOR SEQ ID NO:5:

50 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 482 amino acids  
(B) TYPE: amin acid  
(D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: protein



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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

5 Met Ala Gln Thr Gln Gly Thr Arg Arg Lys Val Cys Tyr Tyr Tyr Asp  
 1 5 10 15  
 Gly Asp Val Gly Asn Tyr Tyr Tyr Gly Gln Gly His Pro Met Lys Pro  
 20 25 30  
 10 His Arg Ile Arg Met Thr His Asn Leu Leu Leu Asn Tyr Gly Leu Tyr  
 35 40 45  
 Arg Lys Met Glu Ile Tyr Arg Pro His Lys Ala Asn Ala Glu Glu Met  
 50 55 60  
 15 Thr Lys Tyr His Ser Asp Asp Tyr Ile Lys Phe Leu Arg Ser Ile Arg  
 65 70 75 80  
 Pro Asp Asn Met Ser Glu Tyr Ser Lys Gln Met Gln Arg Phe Asn Val  
 85 90 95  
 Gly Glu Asp Cys Pro Val Phe Asp Gly Leu Phe Glu Phe Cys Gln Leu  
 100 105 110  
 25 Ser Thr Gly Gly Ser Val Ala Ser Ala Val Lys Leu Asn Lys Gln Gln  
 115 120 125  
 Thr Asp Ile Ala Val Asn Trp Ala Gly Gly Leu His His Ala Lys Lys  
 130 135 140  
 30 Ser Glu Ala Ser Gly Phe Cys Tyr Val Asn Asp Ile Val Leu Ala Ile  
 145 150 155 160  
 Leu Glu Leu Leu Lys Tyr His Gln Arg Val Leu Tyr Ile Asp Ile Asp  
 165 170 175  
 Ile His His Gly Asp Gly Val Glu Glu Ala Phe Tyr Thr Thr Asp Arg  
 180 185 190  
 40 Val Met Thr Val Ser Phe His Lys Tyr Gly Glu Tyr Phe Pro Gly Thr  
 195 200 205  
 Gly Asp Leu Arg Asp Ile Gly Ala Gly Lys Gly Lys Tyr Tyr Ala Val  
 210 215 220  
 45 Asn Tyr Pro Leu Arg Asp Gly Ile Asp Asp Glu Ser Tyr Glu Ala Ile  
 225 230 235 240  
 Phe Lys Pro Val Met Ser Lys Val Met Glu Met Phe Gln Pro Ser Ala  
 245 250 255  
 50 Val Val Leu Gln Cys Gly Ser Asp Ser Leu Ser Gly Asp Arg Leu Gly  
 260 265 270  
 55 Cys Phe Asn Leu Thr Ile Lys Gly His Ala Lys Cys Val Glu Phe Val

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	275	280	285
	Lys Ser Phe Asn Leu Pro Met Leu Met Leu Gly Gly Gly Gly Tyr Thr		
	290	295	300
5	Ile Arg Asn Val Ala Arg Cys Trp Thr Tyr Glu Thr Ala Val Ala Leu		
	305	310	315 320
10	Asp Thr Glu Ile Pro Asn Glu Leu Pro Tyr Asn Asp Tyr Phe Glu Tyr		
		325 330	335
	Phe Gly Pro Asp Phe Lys Leu His Ile Ser Pro Ser Asn Met Thr Asn		
		340 345	350
15	Gln Asn Thr Asn Glu Tyr Leu Glu Lys Ile Lys Gln Arg Leu Phe Glu		
		355 360	365
	Asn Leu Arg Met Leu Pro His Ala Pro Gly Val Gln Met Gln Ala Ile		
		370 375	380
20	Pro Glu Asp Ala Ile Pro Glu Glu Ser Gly Asp Glu Asp Glu Asp Asp		
		385 390	395 400
	Pro Asp Lys Arg Ile Ser Ile Cys Ser Ser Asp Lys Arg Ile Ala Cys		
25		405 410	415
	Glu Glu Glu Phe Ser Asp Ser Glu Glu Glu Gly Glu Gly Gly Arg Lys		
		420 425	430
30	Asn Ser Ser Asn Phe Lys Lys Ala Lys Arg Val Lys Thr Glu Asp Glu		
		435 440	445
	Lys Glu Lys Asp Pro Glu Glu Lys Lys Glu Val Thr Glu Glu Glu Lys		
		450 455	460
35	Thr Lys Glu Glu Lys Pro Glu Ala Lys Gly Val Lys Glu Glu Val Lys		
		465 470	475 480
40	Leu Ala		

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 133 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

- 50 (v) FRAGMENT TYPE: internal

- 55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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1 Ile Asp Phe Leu Gln Arg Val Ser Pro Thr Asn Met Gln Gly Phe Thr  
 5 10 15  
 5 Lys Ser Leu Asn Ala Phe Asn Val Gly Asp Asp Cys Pro Val Phe Pro  
 20 25 30  
 Gly Leu Phe Glu Phe Cys Ser Arg Tyr Thr Gly Ala Ser Leu Gln Gly  
 35 40 45  
 10 Ala Thr Gln Leu Asn Asn Lys Ile Cys Asp Ile Ala Ile Asn Trp Ala  
 50 55 60  
 Gly Gly Leu His His Ala Lys Lys Phe Glu Ala Ser Gly Phe Cys Tyr  
 65 70 75 80  
 15 Val Asn Asp Ile Val Phe Gly Ile Leu Glu Leu Leu Lys Tyr His Pro  
 85 90 95  
 20 Arg Val Leu Tyr Ile Asp Ile Asp Ile His His Gly Asp Gly Val Gln  
 100 105 110  
 Glu Ala Phe Tyr Leu Thr Asp Arg Val Met Thr Val Ser Phe Pro Gln  
 115 120 125  
 25 Ile Arg Glu Ile Tyr  
 130

## (2) INFORMATION FOR SEQ ID NO:7:

30 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 125 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: peptide  
 (v) FRAGMENT TYPE: internal

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

45 Tyr Tyr Cys Leu Asn Val Pro Leu Arg Met Gly Ile Asp Asp Gln Ser  
 1 5 10 15  
 Tyr Lys His Leu Phe Gln Pro Val Ile Asn Gln Val Val Asp Phe Tyr  
 20 25 30  
 50 Gln Pro Thr Cys Ile Val Leu Gln Cys Gly Ala Asp Ser Leu Gly Cys  
 35 40 45  
 Asp Arg Leu Gly Cys Phe Asn Leu Ser Ile Arg Gly His Gly Glu Cys  
 50 55 60  
 55 Val Glu Tyr Val Lys Ser Phe Asn Ile Pro Leu Leu Val Leu Gly Gly

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	65		70		75		80									
	Gly	Gly	Tyr	Thr	Val	Arg	Asn	Val	Ala	Arg	Cys	Trp	Thr	Tyr	Glu	Thr
					85					90					95	
5	Ser	Leu	Leu	Val	Glu	Glu	Ala	Ile	Ser	Glu	Glu	Leu	Pro	Tyr	Ser	Glu
				100					105					110		
10	Tyr	Phe	Glu	Tyr	Phe	Ala	Pro	Asp	Phe	Thr	Leu	His	Pro			
			115					120					125			

## (2) INFORMATION FOR SEQ ID NO:8:

15 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 80 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: peptide  
 (v) FRAGMENT TYPE: internal

## 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

	Asn	Leu	Leu	Val	Leu	Gly	His	Ala	Lys	Cys	Val	Glu	Val	Val	Lys	Thr
	1				5					10					15	
30	Phe	Asn	Leu	Pro	Leu	Leu	Met	Leu	Gly	Gly	Gly	Gly	Tyr	Thr	Ile	Arg
				20					25					30		
	Asn	Val	Ala	Arg	Cys	Trp	Thr	Tyr	Glu	Thr	Ala	Val	Ala	Leu	Asp	Cys
35			35					40					45			
	Glu	Ile	Pro	Asn	Glu	Leu	Pro	Tyr	Asn	Asp	Tyr	Phe	Glu	Tyr	Phe	Gly
		50					55					60				
40	Pro	Asp	Phe	Lys	Leu	His	Ile	Ser	Pro	Ser	Asn	Met	Thr	Asn	Gln	Asn
	65					70					75					80

## (2) INFORMATION FOR SEQ ID NO:11:

45 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1275 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: both  
 (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

55 (ix) FEATURE:  
 (A) NAME/KEY: CDS

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(B) LOCATION: 1..1275

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

5	ATG GCC GAC AAG GAA GCA GCC TTC GAC GAC GCA GTG GAA GAA CGA GTG	48
	Met Ala Asp Lys Glu Ala Ala Phe Asp Asp Ala Val Glu Glu Arg Val	
	1 5 10 15	
10	ATC AAC GAG GAA TAC AAA ATA TGG AAA AAG AAC ACC CCT TTT CTT TAT	96
	Ile Asn Glu Glu Tyr Lys Ile Trp Lys Lys Asn Thr Pro Phe Leu Tyr	
	20 25 30	
15	GAT TTG GTG ATG ACC CAT GCT CTG GAG TGG CCC AGC CTA ACT GCC CAG	144
	Asp Leu Val Met Thr His Ala Leu Glu Trp Pro Ser Leu Thr Ala Gln	
	35 40 45	
20	TGG CTT CCA GAT GTA ACC AGA CCA GAA GGG AAA GAT TTC AGC ATT CAT	192
	Trp Leu Pro Asp Val Thr Arg Pro Glu Gly Lys Asp Phe Ser Ile His	
	50 55 60	
25	CGA CTT GTC CTG GGG ACA CAC ACA TCG GAT GAA CAA AAC CAT CTT GTT	240
	Arg Leu Val Leu Gly Thr His Thr Ser Asp Glu Gln Asn His Leu Val	
	65 70 75 80	
30	ATA GCC AGT GTG CAG CTC CCT AAT GAT GAT GCT CAG TTT GAT GCG TCA	288
	Ile Ala Ser Val Gln Leu Pro Asn Asp Asp Ala Gln Phe Asp Ala Ser	
	85 90 95	
35	CAC TAC GAC AGT GAG AAA GGA GAA TTT GGA GGT TTT GGT TCA GTT AGT	336
	His Tyr Asp Ser Glu Lys Gly Glu Phe Gly Gly Phe Gly Ser Val Ser	
	100 105 110	
40	GGA AAA ATT GAA ATA GAA ATC AAG ATC AAC CAT GAA GGA GAA GTA AAC	384
	Gly Lys Ile Glu Ile Glu Ile Lys Ile Asn His Glu Gly Glu Val Asn	
	115 120 125	
45	AGG GCC CGT TAT ATG CCC CAG AAC CCT TGT ATC ATC GCA ACA AAG ACT	432
	Arg Ala Arg Tyr Met Pro Gln Asn Pro Cys Ile Ile Ala Thr Lys Thr	
	130 135 140	
50	CCT TCC AGT GAT GTT CTT GTC TTT GAC TAT ACA AAA CAT CCT TCT AAA	480
	Pro Ser Ser Asp Val Leu Val Phe Asp Tyr Thr Lys His Pro Ser Lys	
	145 150 155 160	
55	CCA GAT CCT TCT GGA GAG TGC AAC CCA GAC TTG CGT CTC CGT GGA CAT	528
	Pro Asp Pro Ser Gly Glu Cys Asn Pro Asp Leu Arg Leu Arg Gly His	
	165 170 175	
60	CAG AAG GAA GGC TAT GGG CTT TCT TGG AAC CCA AAT CTC AGT GGG CAC	576
	Gln Lys Glu Gly Tyr Gly Leu Ser Trp Asn Pro Asn Leu Ser Gly His	
	180 185 190	
65	TTA CTT AGT GCT TCA GAT GAC CAT ACC ATC TGC CTG TGG GAC ATC AGT	624
	Leu Leu Ser Ala Ser Asp Asp His Thr Ile Cys Leu Trp Asp Ile Ser	



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	195	200	205	
5	GCC GTT CCA AAG GAG GGA AAA GTG GTA GAT GCG AAG ACC ATC TTT ACA Ala Val Pro Lys Glu Gly Lys Val Val Asp Ala Lys Thr Ile Phe Thr 210 215 220			672
10	GGG CAT ACG GCA GTA GTA GAA GAT GTT TCC TGG CAT CTA CTC CAT GAG Gly His Thr Ala Val Val Glu Asp Val Ser Trp His Leu Leu His Glu 225 230 235 240			720
	TCT CTG TTT GGG TCA GTT GCT GAT GAT CAG AAA CTT ATG ATT TGG GAT Ser Leu Phe Gly Ser Val Ala Asp Asp Gln Lys Leu Met Ile Trp Asp 245 250 255			768
15	ACT CGT TCA AAC AAT ACT TCC AAA CCA AGC CAC TCA GTT GAT GCT CAC Thr Arg Ser Asn Asn Thr Ser Lys Pro Ser His Ser Val Asp Ala His 260 265 270			816
20	ACT GCT GAA GTG AAC TGC CTT TCT TTC AAT CCT TAT AGT GAG TTC ATT Thr Ala Glu Val Asn Cys Leu Ser Phe Asn Pro Tyr Ser Glu Phe Ile 275 280 285			864
25	CTT GCC ACA GGA TCA GCT GAC AAG ACT GTT GCC TTG TGG GAT CTG AGA Leu Ala Thr Gly Ser Ala Asp Lys Thr Val Ala Leu Trp Asp Leu Arg 290 295 300			912
30	AAT CTG AAA CTT AAG TTG CAT TCC TTT GAG TCA CAT AAG GAT GAA ATA Asn Leu Lys Leu Lys Leu His Ser Phe Glu Ser His Lys Asp Glu Ile 305 310 315 320			960
	TTC CAG GTT CAG TGG TCA CCT CAC AAT GAG ACT ATT TTA GCT TCC AGT Phe Gln Val Gln Trp Ser Pro His Asn Glu Thr Ile Leu Ala Ser Ser 325 330 335			1008
35	GGT ACT GAT CGC AGA CTG AAT GTC TGG GAT TTA AGT AAA ATT GGA GAG Gly Thr Asp Arg Arg Leu Asn Val Trp Asp Leu Ser Lys Ile Gly Glu 340 345 350			1056
40	GAA CAA TCC CCA GAA GAT GCA GAA GAC GGG CCA CCA GAG TTG TTG TTT Glu Gln Ser Pro Glu Asp Ala Glu Asp Gly Pro Pro Glu Leu Leu Phe 355 360 365			1104
45	ATT CAT GGT GGT CAT ACT GCC AAG ATA TCT GAT TTC TCC TGG AAT CCC Ile His Gly Gly His Thr Ala Lys Ile Ser Asp Phe Ser Trp Asn Pro 370 375 380			1152
50	AAT GAA CCT TGG GTG ATT TGT TCT GTA TCA GAA GAC AAT ATC ATG CAA Asn Glu Pro Trp Val Ile Cys Ser Val Ser Glu Asp Asn Ile Met Gln 385 390 395 400			1200
	GTG TGG CAA ATG GCA GAG AAC ATT TAT AAT GAT GAA GAC CCT GAA GGA Val Trp Gln Met Ala Glu Asn Ile Tyr Asn Asp Glu Asp Pro Glu Gly 405 410 415			1248
55	AGC GTG GAT CCA GAA GGA CAA GGG TCC TAG			1278

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Ser Val Asp Pro Glu Gly Gln Gly Ser  
420 425

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 69 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Asp Xaa Xaa Xaa Asn Xaa Xaa Gly Gly Leu His His Ala Lys Lys Xaa  
1 5 10 15

Glu Ala Ser Gly Phe Cys Tyr Xaa Asn Asp Ile Val Xaa Xaa Ile Xaa  
20 25 30

Glu Leu Leu Xaa Tyr His Xaa Arg Val Xaa Tyr Ile Asp Xaa Asp Xaa  
25 35 40 45

His His Gly Asp Gly Xaa Glu Glu Ala Phe Tyr Xaa Thr Asp Arg Val  
50 55 60

Met Thr Xaa Ser Phe  
65

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 69 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Asp Ile Ala Xaa Asn Trp Ala Gly Gly Leu His His Ala Lys Lys Xaa  
1 5 10 15

Glu Ala Ser Gly Phe Cys Tyr Val Asn Asp Ile Val Xaa Xaa Ile Leu  
20 25 30

Glu Leu Leu Lys Tyr His Xaa Arg Val Leu Tyr Ile Asp Ile Asp Ile  
35 40 45

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His His Gly Asp Gly Xaa Glu Glu Ala Phe Tyr Xaa Thr Asp Arg Val  
 50 55 60

5 Met Thr Val Ser Phe  
 65

## (2) INFORMATION FOR SEQ ID NO:14:

10 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 33 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: peptide  
 (v) FRAGMENT TYPE: internal

## 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Cys Val Xaa Xaa Xaa Lys Xaa Phe Xaa Xaa Pro Xaa Xaa Xaa Xaa Gly  
 1 5 10 15

25 Gly Gly Gly Tyr Thr Xaa Arg Asn Val Ala Arg Xaa Trp Xaa Xaa Glu  
 20 25 30

Thr

## 30 (2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 33 amino acids  
 (B) TYPE: amino acid  
 35 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

40 (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

45 Cys Val Glu Xaa Val Lys Xaa Phe Asn Xaa Pro Leu Leu Xaa Leu Gly  
 1 5 10 15

Gly Gly Gly Tyr Thr Xaa Arg Asn Val Ala Arg Cys Trp Thr Tyr Glu  
 20 25 30

50 Thr

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What is claimed is:

1. An isolated or recombinant *HDx* polypeptide.
2. The polypeptide of claim 1, of mammalian origin.
3. The polypeptide of claim 3, of human origin.
- 5 4. The polypeptide of claim 1, which polypeptide comprises an *HDx* polypeptide sequence at least 88 percent homologous with SEQ ID No: 2, or fragment thereof.
5. The polypeptide of claim 1, which polypeptide comprises an *HDx* polypeptide sequence at least 95 percent homologous with SEQ ID No: 2, or fragment thereof.
- 10 6. The polypeptide of claim 1, which polypeptide comprises an *HDx* polypeptide sequence designated in SEQ ID No: 2.
7. The polypeptide of claim 1, which polypeptide is encoded by a nucleic acid having a coding sequence, or portion thereof, which hybridizes under stringent conditions to the nucleic acid designated in SEQ ID No. 1.
9. The polypeptide of claim 1, which polypeptide is an acetylase activity.
- 15 10. The polypeptide of claim 1, which polypeptide binds to an RbAp48 protein.
11. The polypeptide of claim 1, which polypeptide is a fusion protein.
12. The polypeptide of claim 1, which polypeptide has a molecular weight in the range of 45-70 Kd.
- 20 13. An isolated or recombinant polypeptide comprising an *HDx* polypeptide sequence homologous or identical SEQ ID No. 2, or a fragment thereof which retains one or more of (i) a histone deacetylase activity, (ii) a histone binding activity and (iii) an RbAp48 binding activity.
- 25 14. The polypeptide of claim 13, which polypeptide comprises an *HDx* sequence represented in the general formula  
DXXNXGGLHHAKKXEASGFCYXNDIVXXI-  
XELLXYHXR VXYIDXD XHHGDGX EAFYXTDR VMTXSF.
15. The polypeptide of claim 13, which polypeptide comprises an *HDx* sequence represented in the general formula  
CVXXXKXFXXPXXXG GGGGYTXRNVARX-WXXET.
- 30 16. The polypeptide of claim 13, which polypeptide deacetylates acetylated histones.

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17. The polypeptide of claim 13, which polypeptide is a dominant negative inhibitor which antagonizes deacetylation of acetylated histones.
18. The polypeptide of claim 13, which polypeptide comprises an *HDx* sequence at least 88 percent homologous with SEQ ID No: 2, or fragment thereof.
- 5 19. The polypeptide of claim 13, which polypeptide comprises an *HDx* sequence at least 95 percent homologous with SEQ ID No: 2, or fragment thereof.
20. The polypeptide of claim 13, which polypeptide includes at least 25 amino acid residues of an *HDx* polypeptide sequence.
- 10 21. The polypeptide of claim 13, wherein said polypeptide modulates cellular proliferation.
22. An isolated or recombinant polypeptide comprising an *HDx* polypeptide sequence represented SEQ ID No. 2, or a fragment thereof which retains one or more of (i) a histone deacetylase activity, (ii) a histone binding activity and (iii) an RbAp48 binding activity.
- 15 23. The polypeptide of claim 22, wherein said fusion protein includes, as a second polypeptide sequence, a polypeptide which functions as a detectable label for detecting the presence of said fusion protein or as a matrix-binding domain for immobilizing said fusion protein.
- 20 24. The polypeptide of claim 13, wherein said polypeptide is a fusion protein further comprising, in addition to said *HDx* sequence, a second polypeptide sequence having an amino acid sequence unrelated to an *HDx* polypeptide sequence.
25. A purified or recombinant *HDx* polypeptide encoded by a nucleic acid which hybridizes under stringent conditions to a nucleotide sequence designated in SEQ ID No. 1.
- 25 26. A purified or recombinant *HDx* polypeptide comprising a  $\nu$  motif represented in the general formula  

$$\text{DIAX}_1\text{NWAGGLHHAKKX}_2\text{EASGFCYVNDIVX}_3\text{X}_4\text{ILELLKYH-}$$

$$\text{X}_5\text{RVLYIDIDHHGDGX}_6\text{EAFYX}_7\text{TDRVMTVSF}$$
 and a  $\chi$  motif represented in  

$$\text{CVEX}_1\text{VKX}_2\text{FNX}_3\text{P-X}_4\text{LX}_5\text{LGGGGYTX}_6\text{RNVARCWTYET}.$$
- 30 27. An isolated nucleic acid which encodes a deacetylase activity and hybridizes under stringent conditions to a nucleotide sequence designated in SEQ ID No. 1.



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28. An isolated nucleic acid encoding an *HDx* polypeptide, which polypeptide specifically modulates histone acetylation.
29. The nucleic acid of claim 28, which *HDx* polypeptide comprises a  $\nu$  motif represented in the general formula  
5      $\text{DIAX}_1\text{NWAGGLHHAKKX}_2\text{EASGFCYVNDIVX}_3\text{X}_4\text{ILELL-}$   
 $\text{KYHX}_5\text{RVLYIDIDIHHGDGX}_6\text{EAFYX}_7\text{TDRVMTVSF}$  and a  $\chi$  motif represented in  $\text{CVEX}_1\text{VKX}_2\text{FNX}_3\text{P-X}_4\text{LX}_5\text{LGGGGYTX}_6\text{RNVARCWTYET}$ .
30. The nucleic acid of claim 28, which *HDx* polypeptide comprises a polypeptide sequence at least 88 percent homologous with SEQ ID No: 2, or fragment thereof.
- 10     31. The nucleic acid of claim 28, which *HDx* polypeptide comprises a polypeptide sequence at least 95 percent homologous with SEQ ID No: 2, or fragment thereof.
32. The nucleic acid of claim 28, which *HDx* polypeptide comprises a polypeptide sequence designated in SEQ ID No: 2.
- 15     33. The nucleic acid of claim 28, which *HDx* polypeptide has a molecular weight in the range of 45-70 Kd.
34. The nucleic acid of claim 28, which *HDx* polypeptide is a fusion protein further comprising, in addition to *HDx* polypeptide sequences, a second polypeptide sequence having an amino acid sequence unrelated to a nucleic acid sequence.
- 20     35. The nucleic acid of claim 34, wherein said fusion protein includes, as a second polypeptide sequence, a polypeptide which functions as a detectable label for detecting the presence of said fusion protein or as a matrix-binding domain for immobilizing said fusion protein.
- 25     36. The nucleic acid of claim 28, further comprising a transcriptional regulatory sequence operably linked to said nucleotide sequence so as to render said nucleic acid suitable for use as an expression vector.
37. An expression vector, capable of replicating in at least one of a prokaryotic cell and eukaryotic cell, comprising the nucleic acid of claim 36.
38. A host cell transfected with the expression vector of claim 37 and expressing said recombinant polypeptide.
- 30     39. A method of producing a recombinant *HDx* polypeptide comprising culturing the cell of claim 38 in a cell culture medium to express said recombinant polypeptide and isolating said recombinant polypeptide from said cell culture.

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40. A transgenic non-human animal having cells which harbor a heterologous transgene encoding an *HDx* polypeptide.
41. A transgenic non-human animal having cells in which an *HDx* gene is disrupted.
42. A recombinant transfection system, comprising
- 5 (i) a gene construct including the nucleic acid of claim 28 and operably linked to a transcriptional regulatory sequence for causing expression of said *HDx* polypeptide in eukaryotic cells, and
- (ii) a gene delivery composition for delivering said gene construct to a cell and causing the cell to be transfected with said gene construct.
- 10 43. The recombinant transfection system of claim 42, wherein the gene delivery composition is selected from a group consisting of a recombinant viral particle, a liposome, and a poly-cationic nucleic acid binding agent.
44. A nucleic acid composition comprising a substantially purified oligonucleotide, said oligonucleotide including a region of nucleotide sequence which hybridizes under
- 15 stringent conditions to at least 25 consecutive nucleotides of sense or antisense sequence of an *HDx* gene.
45. The nucleic acid composition of claim 44, which oligonucleotide hybridizes under stringent conditions to at least 50 consecutive nucleotides of sense or antisense sequence of an *HDx* gene.
- 20 46. The nucleic acid composition of claim 44, wherein said oligonucleotide further comprises a label group attached thereto and able to be detected.
47. The nucleic acid composition of claim 44, wherein said oligonucleotide has at least one non-hydrolyzable bond between two adjacent nucleotide subunits.
48. A test kit for detecting cells which contain an *HDx*-encoding nucleic acid,
- 25 comprising the nucleic acid composition of claim 44 for measuring, in a sample of cells, a level of nucleic acid encoding an *HDx* protein.
49. A method for modulating one or more of growth, differentiation, or survival of a mammalian cell responsive to *HDx*-mediated histone deacetylation, comprising
- 30 treating the cell with an effective amount of an agent which modulates the deacetylase activity of an *HDx* polypeptide thereby altering, relative to the cell in the absence of the agent, at least one of (i) rate of growth, (ii) differentiation, or (iii) survival of the cell.

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50. An antibody to an *HDx* polypeptide.
51. The antibody of claim 50, wherein said antibody is monoclonal.
52. A diagnostic assay for identifying a cell or cells at risk for a disorder characterized by unwanted cell proliferation or differentiation, comprising detecting, in a cell sample, the presence or absence of a genetic lesion characterized by at least one of (i) aberrant modification or mutation of a gene encoding an *HDx* protein, and (ii) mis-expression of said gene; wherein a wild-type form of said gene encodes an *HDx* protein characterized by an ability to modulate the signal transduction activity of a  $TGF\beta$  receptor.
53. The assay of claim 52, wherein detecting said lesion includes:
- providing a diagnostic probe comprising a nucleic acid including a region of nucleotide sequence which hybridizes to a sense or antisense sequence of said gene, or naturally occurring mutants thereof, or 5' or 3' flanking sequences naturally associated with said gene;
  - combining said probe with nucleic acid of said cell sample; and
  - detecting, by hybridization of said probe to said cellular nucleic acid, the existence of at least one of a deletion of one or more nucleotides from said gene, an addition of one or more nucleotides to said gene, a substitution of one or more nucleotides of said gene, a gross chromosomal rearrangement of all or a portion of said gene, a gross alteration in the level of an mRNA transcript of said gene, or a non-wild type splicing pattern of an mRNA transcript of said gene.
54. The assay of claim 53, wherein hybridization of said probe further comprises subjecting the probe and cellular nucleic acid to a polymerase chain reaction (PCR) and detecting abnormalities in an amplified product.
55. The assay of claim 53, wherein hybridization of said probe further comprises subjecting the probe and cellular nucleic acid to a ligation chain reaction (LCR) and detecting abnormalities in an amplified product.
56. The assay of claim 53, wherein said probe hybridizes under stringent conditions to the nucleic acid designated by SEQ ID No. 1.
57. An assay for screening test compounds to identify agents which inhibit the deacetylation of histones comprising:

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- i. providing a reaction mixture including a histone deacetylase activity of an *HDx*-like polypeptide, a substrate for a histone deacetylase, and a test compound; and
  - ii. detecting the conversion of the substrate to product,
- 5 wherein a statistically significant decrease in the conversion of the substrate in the presence of the test compound is indicative of a potential inhibitor of histone deacetylation.
- 58. The assay of claim 57, wherein the *HDx-like* polypeptide is of mammalian origin.
- 59. The assay of claim 57, wherein the *HDx-like* polypeptide is an RPD3-like
- 10 deacetylase of fungal origin.
- 60. The assay of claim 57, wherein the reaction mixture is a reconstituted protein mixture.
- 61. The assay of claim 57, wherein said reaction mixture is a cell lysate.
- 62. The assay of claim 57, wherein the *HDx-like* polypeptide is a recombinant protein.
- 15 63. An assay for screening test compounds to identify agents which inhibit histone deacetylase interaction with cellular proteins, comprising:
  - i. providing a reaction mixture including an *HDx*-like protein, an *HDx*-binding protein, and a test compound; and
  - ii. detecting the interaction of the *HDx*-like protein and the *HDx* binding
  - 20 protein,wherein a statistically significant decrease in the interaction of the proteins in the presence of the test compound is indicative of a potential inhibitor of a histone deacetylase.
- 64. The assay of claim 63, wherein the *HDx-like* protein is of mammalian origin.
- 25 65. The assay of claim 63, wherein the *HDx-like* polypeptide is an RPD3-like deacetylase of fungal origin.
- 66. The assay of claim 63, wherein the *HDx-like* protein is a histone, or a portion thereof which interacts with an *HDx*-like polypeptide.
- 67. The assay of claim 63, wherein the *HDx-like* protein is an PbAp48 protein, or a
- 30 portion thereof which interacts with an *HDx*-like polypeptide.
- 68. The assay of claim 63, wherein the reaction mixture is a reconstituted protein mixture.

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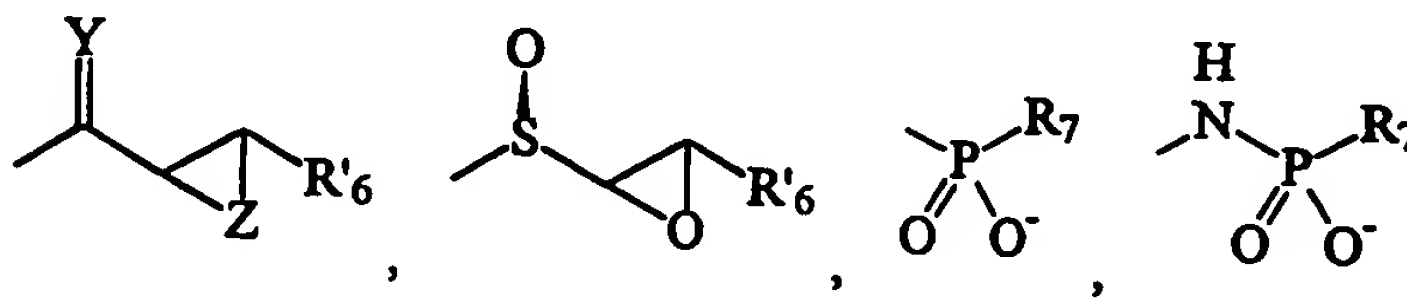
69. The assay of claim 63, wherein said reaction mixture is a cell lysate.
70. The assay of claim 63, wherein the *HDx-like* polypeptide is a recombinant protein.
71. The assay of claim 63, wherein one or both of the *HDx-like* protein and *HDx-binding* protein is a fusion protein.
- 5 72. The assay of claim 63, wherein at least one of the *HDx-like* protein and *HDx-binding* protein comprises an endogenous detectable label for detecting the formation of said complex.
73. The method of claim 63, which reaction mixture is a whole cell, and interaction of the *HDx-like* protein and *HDx-binding* protein is detected in a two hybrid assay system .
- 10 74. A composition for inhibiting a histone deacetylase comprising a compound represented by the general formula A-B-C, wherein

A is selected from the group consisting of cycloalkyls, unsubstituted and substituted aryls, heterocyclyls, amino acyls, and cyclopeptides;

15 B is selected from the group consisting of substituted and unsubstituted C<sub>4</sub>-C<sub>8</sub> alkylidenes, C<sub>4</sub>-C<sub>8</sub> alkenylidenes, C<sub>4</sub>-C<sub>8</sub> alkynylidenes, and -(D-E-F)-, in which D and F are, independently, absent or represent a C<sub>2</sub>-C<sub>7</sub> alkylidene, a C<sub>2</sub>-C<sub>7</sub> alkenylidene or a C<sub>2</sub>-C<sub>7</sub> alkynylidene, and E represents O, S, or NR', in which R' represents H, a lower alkyl, a lower alkenyl, a lower alkynyl, an aralkyl, aryl, or a heterocyclyl; and

20

C is selected from the group consisting of



25 and a boronic acid; in which Z represents O, S, or NR<sub>5</sub>, and Y; R<sub>5</sub> represents a hydrogen, an alkyl, an alkoxycarbonyl, an aryloxycarbonyl, an alkylsulfonyl, an arylsulfonyl or an aryl; R'<sub>6</sub> represents hydrogen, an alkyl, an alkenyl, an alkynyl or an aryl; and R<sub>7</sub> represents a hydrogen, an alkyl, an aryl, an alkoxy, an aryloxy, an amino, a hydroxylamino, an alkoxylamino or a halogen; with the proviso that the compound is not trapoxin.



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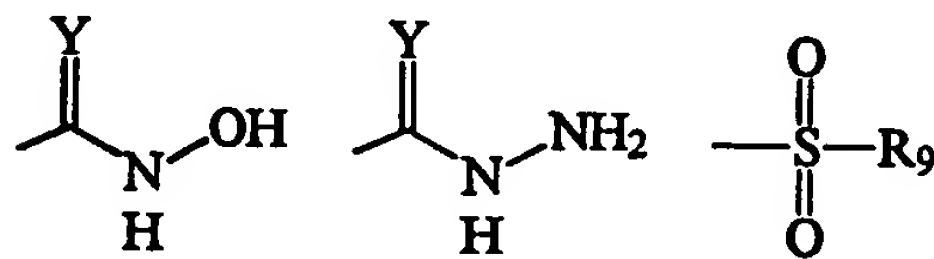
75. A pharmaceutical preparation comprising (i) the composition of claim 74 in an amount effective for inhibiting proliferation of a cell, and (ii) a pharmaceutically acceptable diluent.
- 5 76. A method for modulating one or more of growth, differentiation, or survival of a mammalian cell responsive to *HDx*-mediated histone deacetylation, comprising treating the cell with an effective amount of the composition of claim 74 so as to modulate the deacetylase activity and alter, relative to the cell in the absence of the agent, at least one of (i) the rate of growth, (ii) the differentiation state, or (iii) the rate of survival of the cell.
- 10 77. A composition for inhibiting a histone deacetylase comprising a compound represented by the general formula A-B-C, wherein

15 A is selected from the group consisting of cycloalkyls, unsubstituted and substituted aryls, heterocyclyls, amino acyls, and cyclopeptides;

B is selected from the group consisting of substituted and unsubstituted C<sub>4</sub>-C<sub>8</sub> alkylidenes, C<sub>4</sub>-C<sub>8</sub> alkenylidenes, C<sub>4</sub>-C<sub>8</sub> alkynylidenes, and -(D-E-F)-, in which D and F are, independently, absent or represent C<sub>2</sub>-C<sub>7</sub> alkylidenes, C<sub>2</sub>-C<sub>7</sub> alkenylidenes or C<sub>2</sub>-C<sub>7</sub> alkynylidenes, and E represents O, S, or NR', in which R' represents H, a lower alkyl, a lower alkenyl, a lower alkynyl, an aralkyl, an aryl, or a heterocyclyl; and

20

C is selected from the group consisting of



, in which R<sub>9</sub> represents a hydrogen, an alkyl, an aryl, a hydroxyl, an alkoxy, an aryloxy or an amino,

25 with the proviso that the inhibitor compound is not trichostatin.

78. A pharmaceutical preparation comprising (i) the composition of claim 77 in an amount effective for inhibiting proliferation of a cell, and (ii) a pharmaceutically acceptable diluent.
- 30

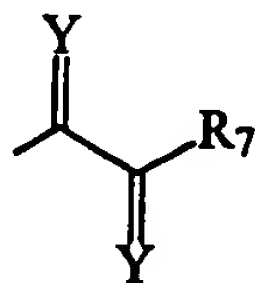
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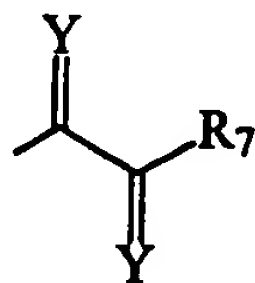
79. A method for modulating one or more of growth, differentiation, or survival of a mammalian cell responsive to *HDx*-mediated histone deacetylation, comprising treating the cell with an effective amount of the composition of claim 77 so as to modulate the deacetylase activity and alter, relative to the cell in the absence of the agent, at least one of (i) the rate of growth, (ii) the differentiation state, or (iii) the rate of survival of the cell.

80. A composition for inhibiting a histone deacetylase comprising a compound represented by the general formula A-B-C, wherein

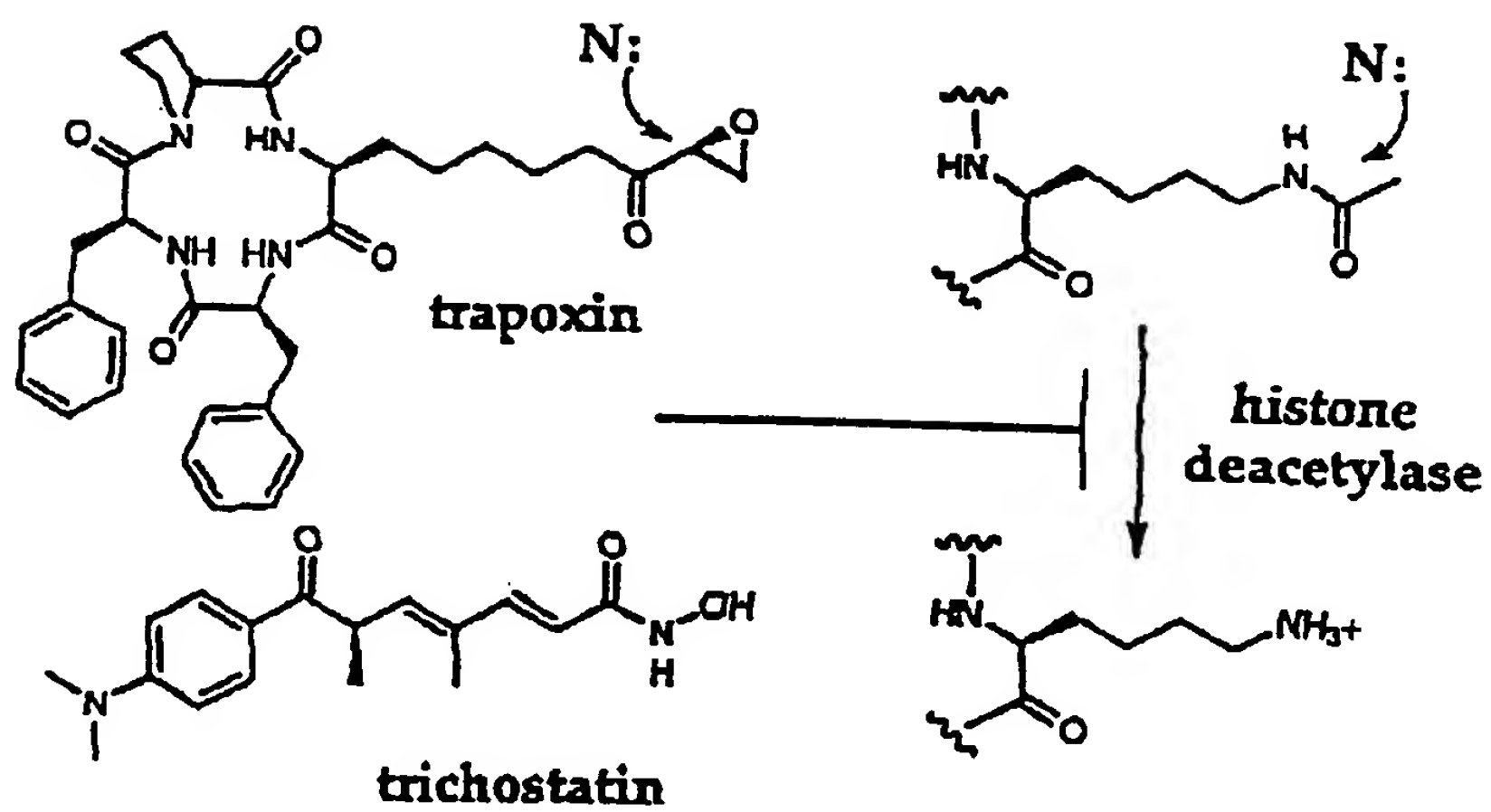
A is selected from the group consisting of cycloalkyls, unsubstituted and substituted aryls, heterocyclyls, amino acyls, and cyclopeptides;

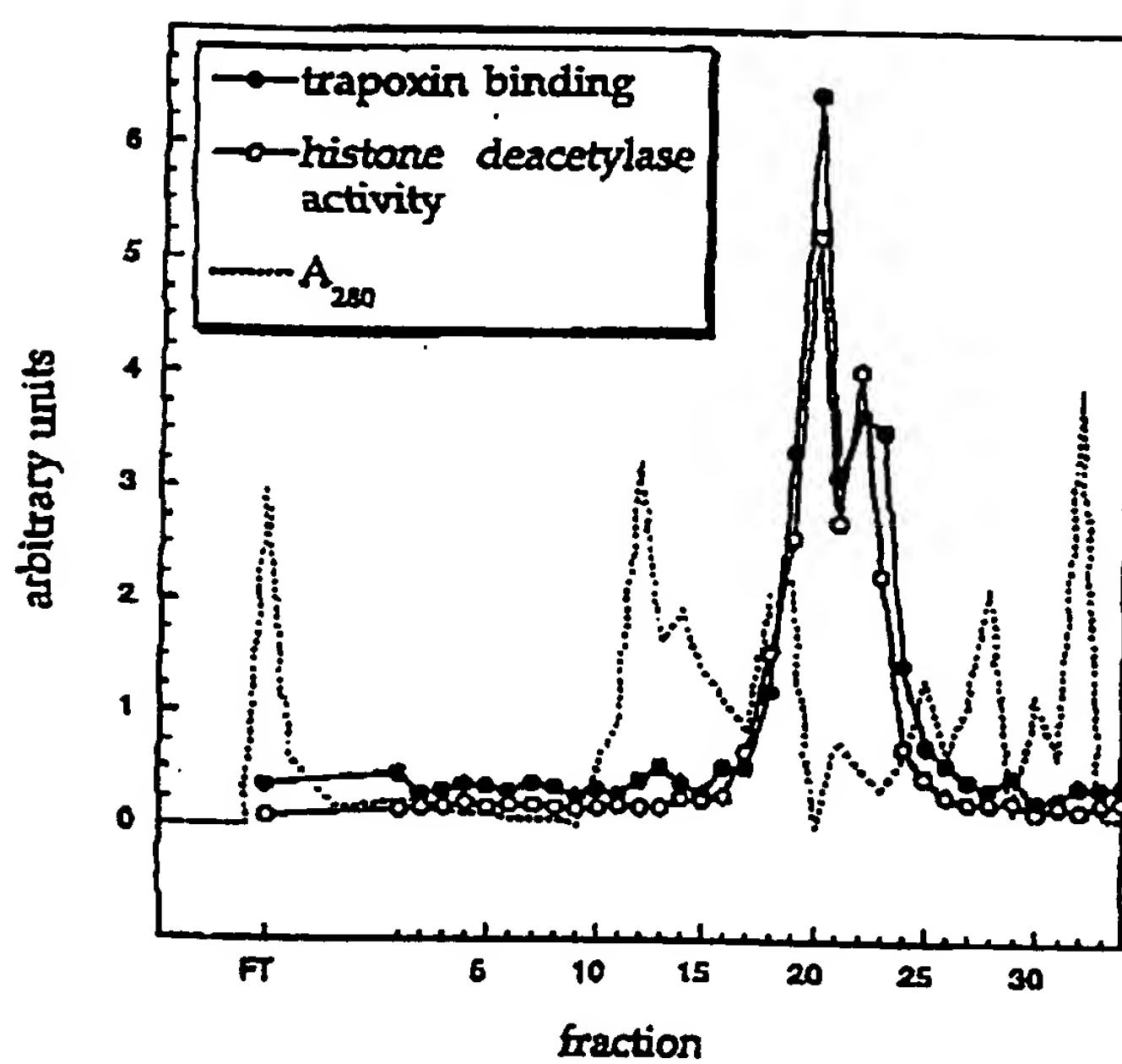
B is selected from the group consisting of substituted and unsubstituted  $C_4$ - $C_8$  alkylidenes,  $C_4$ - $C_8$  alkenylidenes,  $C_4$ - $C_8$  alkynylidenes, and  $-(D-E-F)-$ , in which D and F are, independently, absent or a  $C_2$ - $C_7$  alkylidene, a  $C_2$ - $C_7$  alkenylidene, or a  $C_2$ - $C_7$  alkynylidene, and E represents O, S, or  $NR'$ , in which  $R'$  is H, lower alkyl, lower alkenyl, lower alkynyl, aralkyl, aryl, or heterocyclyl; and



C represents ; in which Y is O or S, and  $R_7$  represents a hydrogen, an alkyl, an aryl, an alkoxy, an aryloxy, an amino, a hydroxylamino, an alkoxyamino or a halogen.

81. A pharmaceutical preparation comprising (i) the composition of claim 80 in an amount effective for inhibiting proliferation of a cell, and (ii) a pharmaceutically acceptable diluent.
82. A method for modulating one or more of growth, differentiation, or survival of a mammalian cell responsive to *HDx*-mediated histone deacetylation, comprising treating the cell with an effective amount of the composition of claim 80 so as to modulate the deacetylase activity and alter, relative to the cell in the absence of the agent, at least one of (i) the rate of growth, (ii) the differentiation state, or (iii) the rate of survival of the cell.

*Figure 1A*

*Figure 1B*

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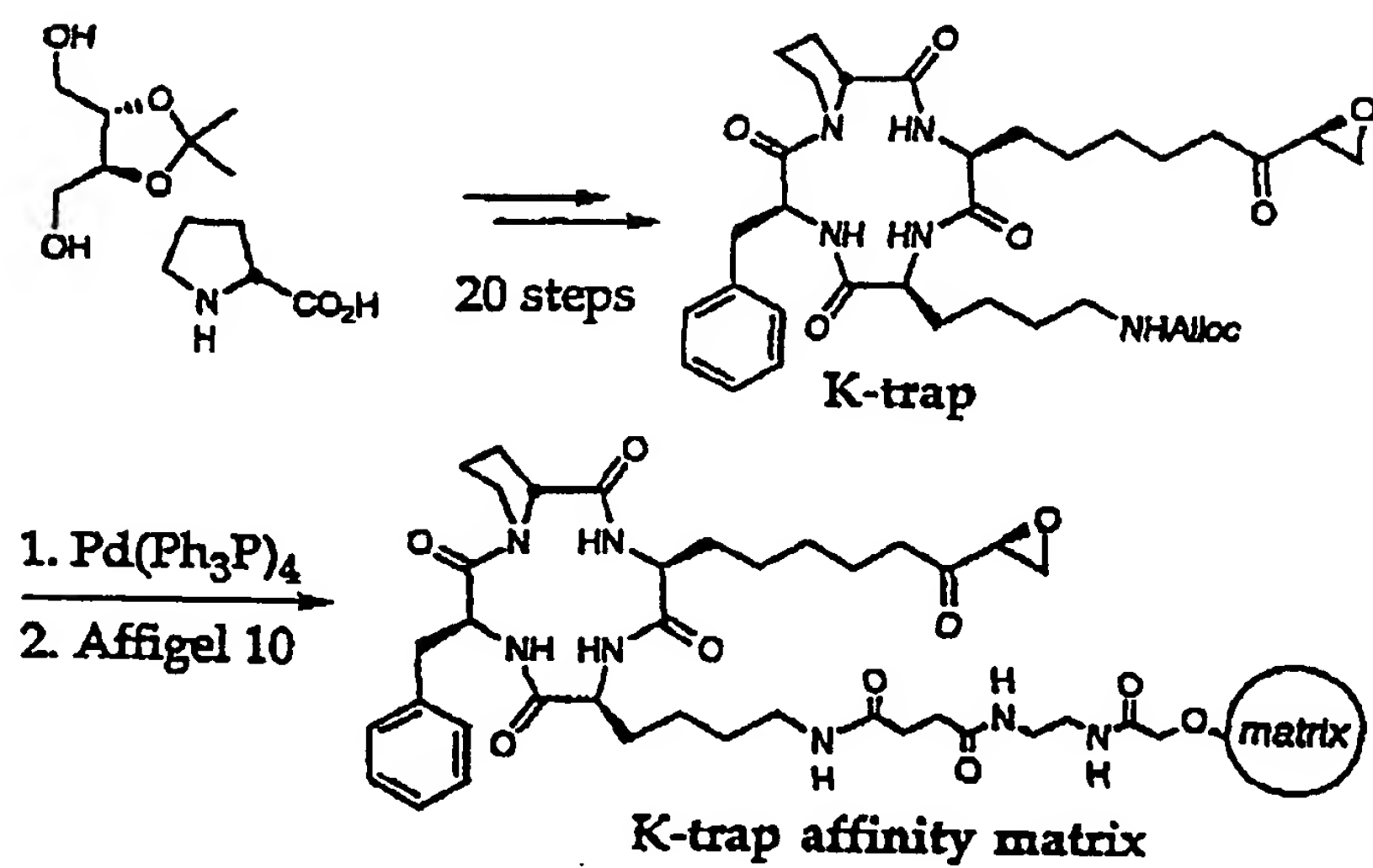
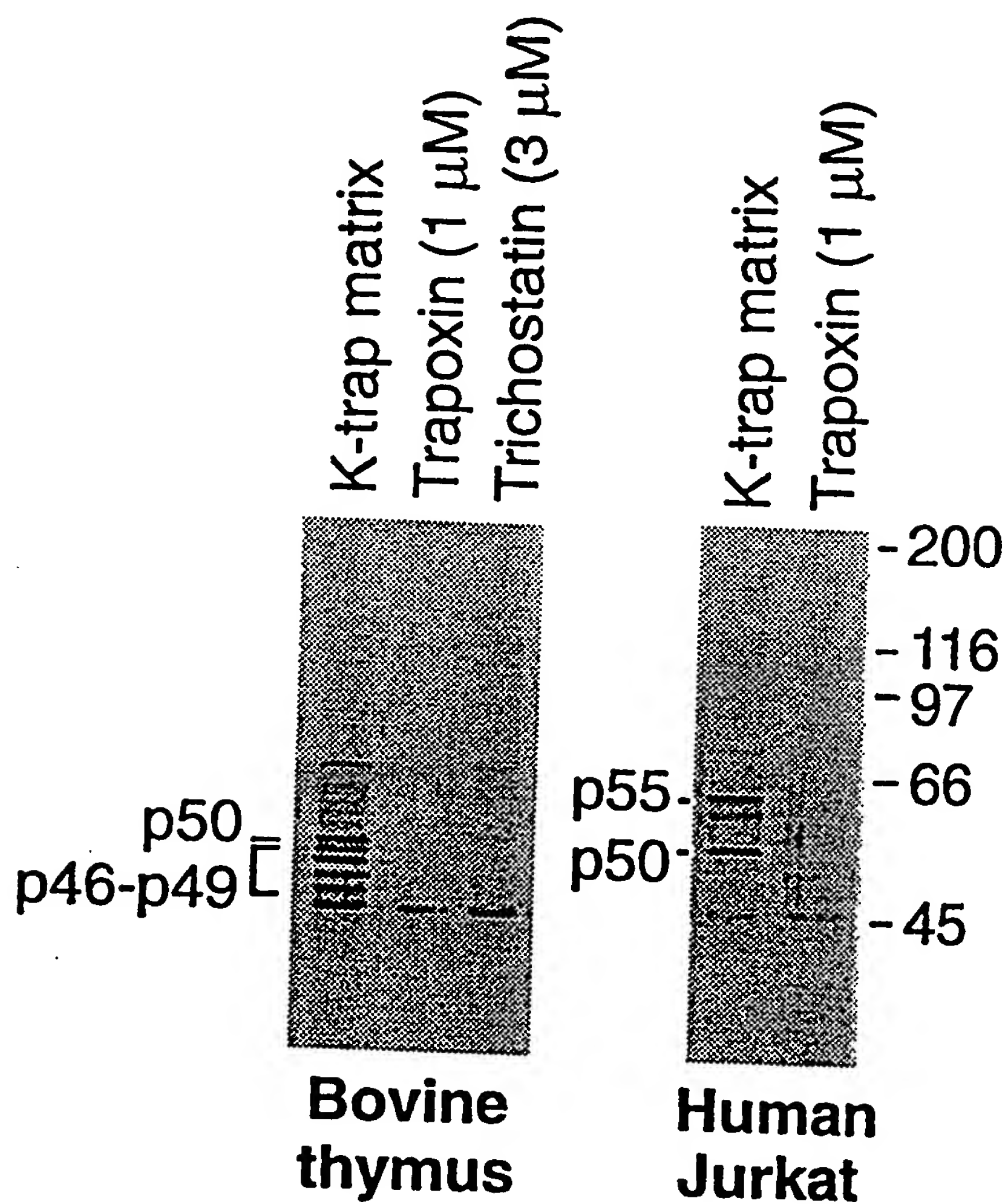
**Figure 2A**



Figure 2B



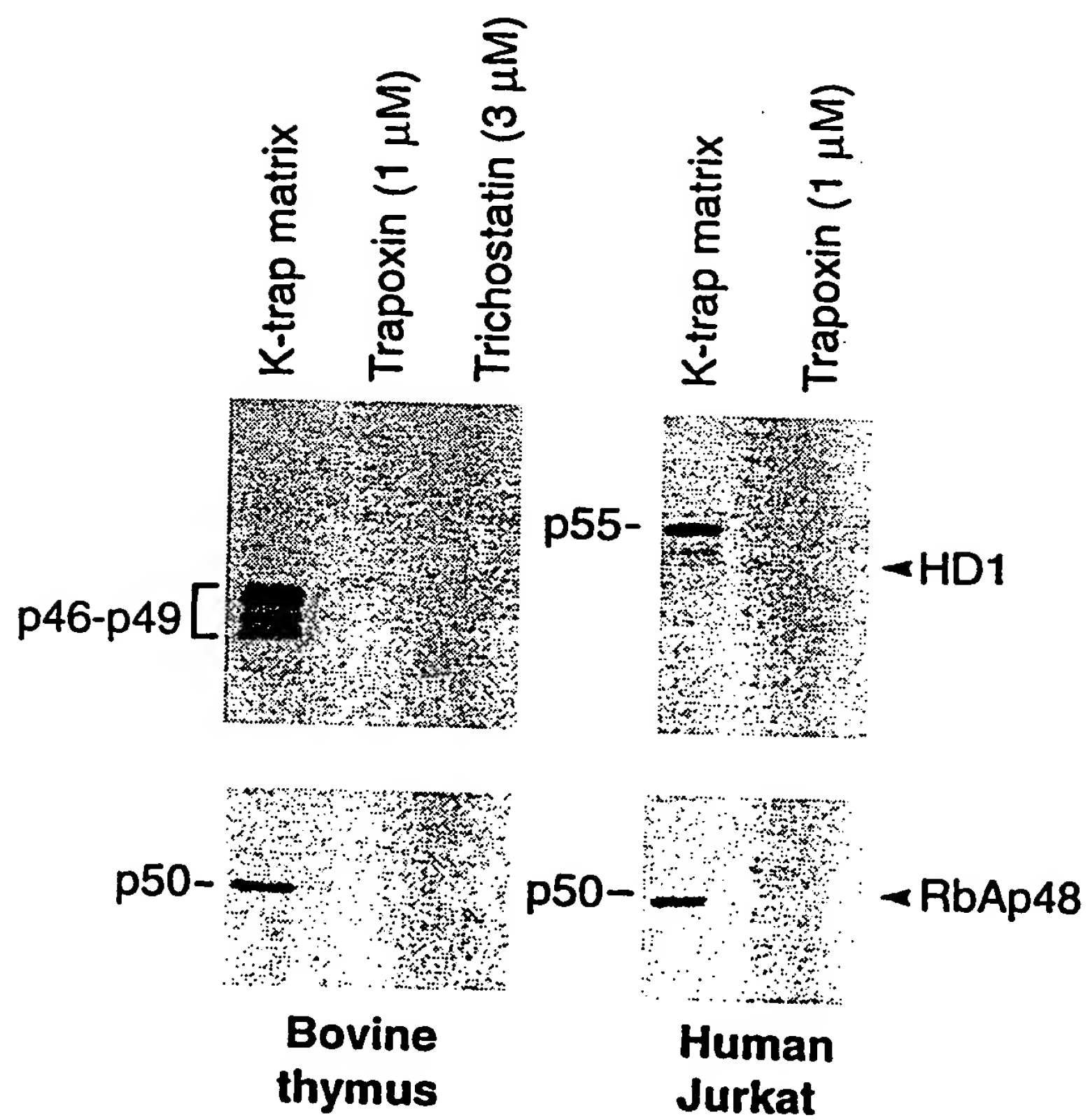
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1 MAQTQGTTRK VCYYXDGVG NYYGQGHM KPHRIRMTN LLLNYGLYRK MBIYRPHKAN ABEMTKYHSD  
71 DYIKFLRSIR PDNMSEYSKQ MQRPNUGEDC PVPDGLFEFC QLSTGGGVAQ AVKLNKQOTD IAVNMAAGGLH  
41 HAKKSEASGF CYVNDIVLAI LELLKXHQV LYIDIDIHG DGVBEAFYTT DRMTVSEHK YGBYFPGTGD  
11 LRDIGAGK GK YYAVNYPLAD GIDDESIEAI FKPVMSKVMH MFQPSAVLQ CGSDSLSGDR LGCFNLTKG  
81 HAKCVBEFVKS FNLPHMLGG GGYTIRNVAR CWTYETAVAL DTEIPNHELX NDYERYFGPD FKLHISPSNM  
51 TNQNTNEYLE KIKQRLFENL RMLPHAPGVQ MQAIPEDAIP BESGDEDED PDKRISICSS DKRIACEEEF  
21 SDSEEBEGGG RKNSSNEFKA KRVRTEDERE KDPREKKEVT EEEKTKKEKP BAKGVKBEVR LA

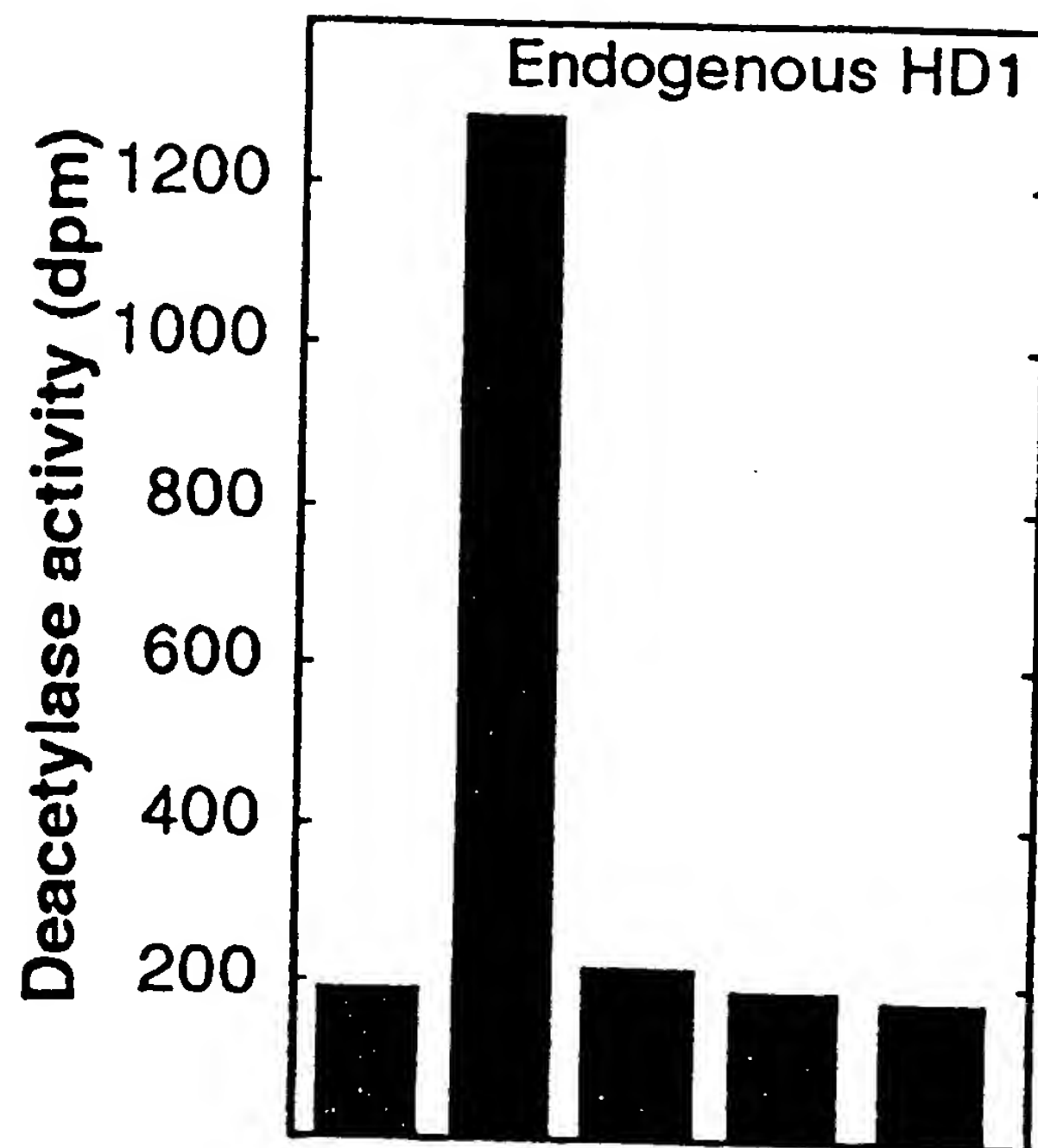
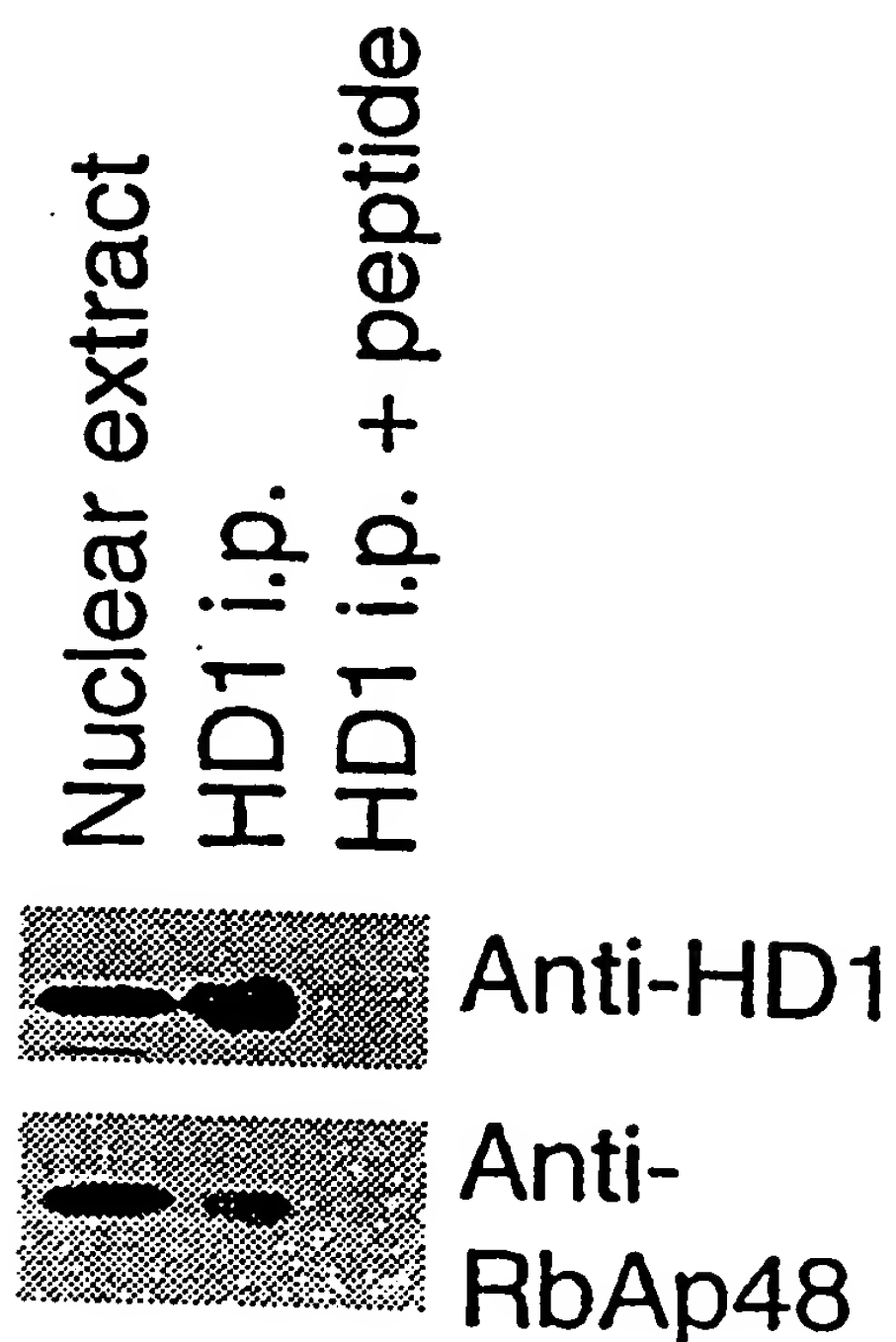
Figure 3A

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*Figure 3B*



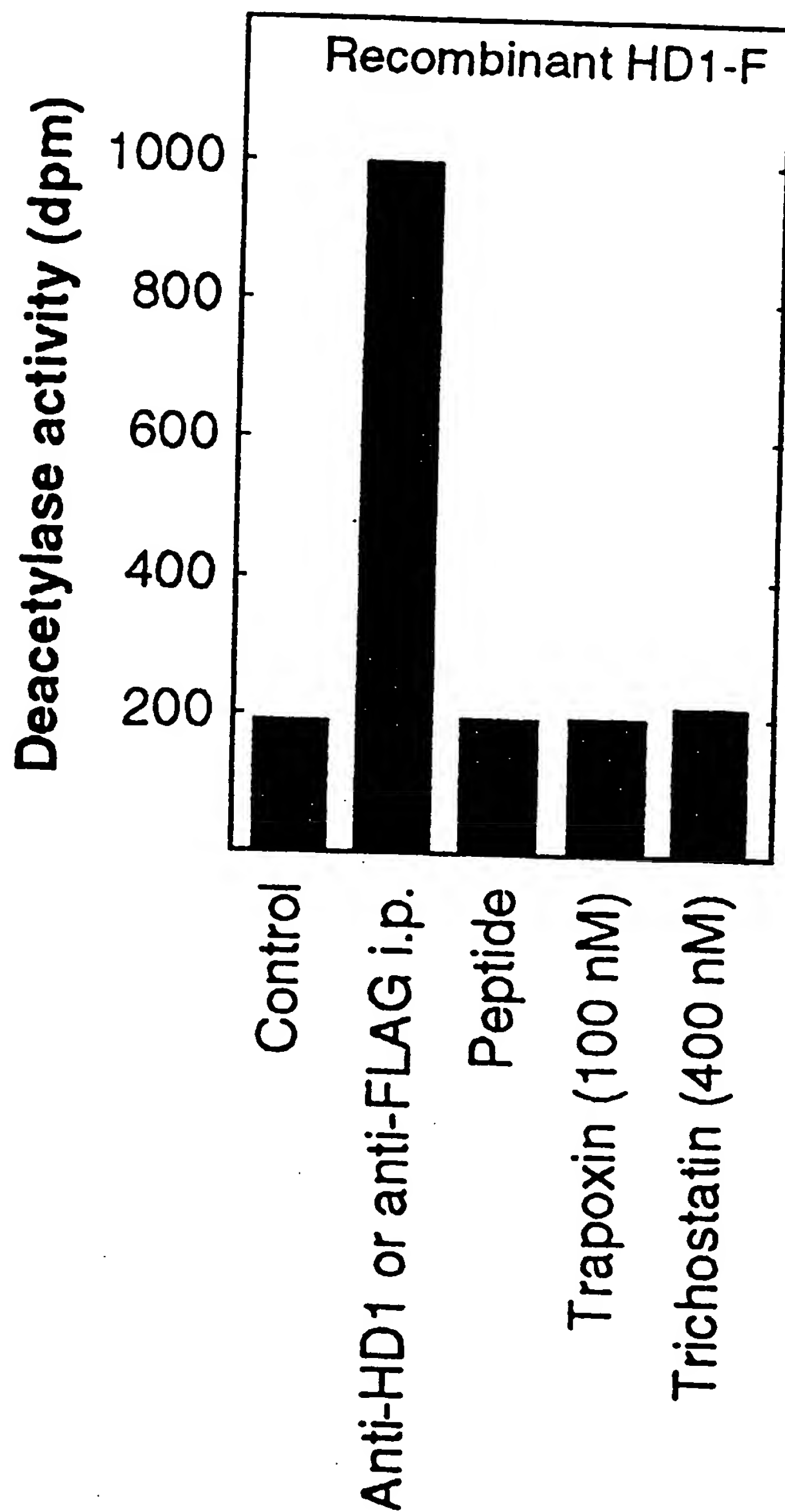
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*Figure 4A**Figure 4B*

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Figure 4C

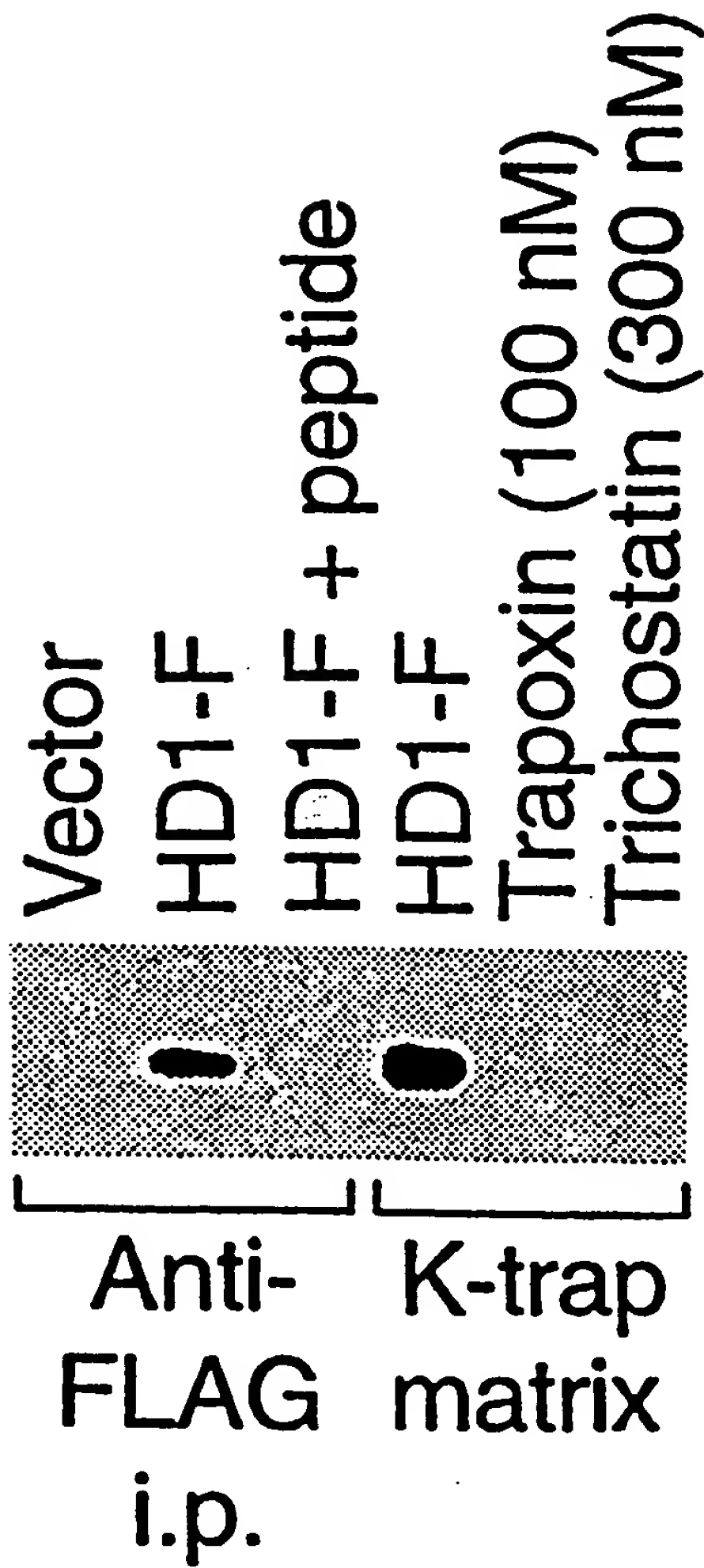


SUBSTITUTE SHEET (RULE 26)



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Figure 4D



*Figure 5A*

## Sequences

1> HD1                    2> HSC11A021                    3> R21136                    4> F07807  
5> R18769                6> D31480                    7> R98879                    8> N59055

1> ATGGCGCAGACgCAGGGCACC CGAGGAAAGTCTGTTACTACTACGACGGGGATGTTGGA 50  
6> ATGGCGCAGACGCAGGGCACC CGAGGAAAGTNTGTTACTACTACGACGGGGATGTTGGA

1> AATTACTATTATGGACAAGGCCACCCAATGAAGCCTCACCGAATCCGCATGACTCATAAT 100  
6> AATTACTATTATGGACAAGGCCACCCAATGAAGCCTCACCGAATCCGCATGACTCATAAT

1> TTGCTGCTCAACTATGGTCTCTACCGAAAAATGGAAATCTATCGCCCTCACAAAGCCAAT 150  
6> TTGCTGCTCAACTATGGTCTCTACCGAAAAATGGAAATCTATCGNCCTCACAAAGCCAAT

1> gCTGAGGAGATGACCAAGTACCACAGCGATGACTACATTAAATTCTTGCGCTCCATCCGT 200  
2> ATTGACTTCCTGCAGAGAGTCAGC  
6> NCTGAGGAGATGACCAAGTANCACAGCGATGAC  
7> TCCTGCAGAGAGTCAGC

1> CCAGATAACATGTCGGAGTACAGCAAGCAGATGCAGAGATTCAACGTTGGTGAGGACTGT 250 300  
2> CCCACCAATATGCAAGGCTTCACCAAGAGTCTTAATGCCTTCAACGTAGGCGATGACTGC  
5> CGATGACTGC  
7> CCCACCAATATGCAAGGCTTCACCAAGAGTCTTAATGCCTTCAACGTAGGCGATGACTGC

1> CCAGTATTCGATGGCCTGTTTGAGTTCTGTCAGTTGTCTACTGGTGGTTCTGTGGCAAGT 359  
2> CCAGTGTTTCCCGGGCTCTTTGAGTTCTGCTCGCGTTACACAGGCGCATCTCTGCAAGGA  
5> CCAGTGTTTCCCGGGCTCTTTGAGTTCTGCTCGCGTTACACAGGCGCATCTCTGCAAGGA  
7> CCAGTGTTTCCCGGGCTCTTTGAGTTCTGCTCGCGTTACACAGGCGCATCTCTGCAAGGA

1> GCTGTGAAACTTAATAAGCAGCAGACGGACATCGCTGTGAATTGGGCTGGGGGGCTGCAC 400  
2> GCAACCCAGCTGAACAACAAGATCTGTGATATTGCCATTAACCTGGGCTGGTGGTCTGCAC  
5> GCAACCCAGCTGAACAACAAGATCTGTGATATTGCCATTAACCTGGGCTGGTGGCTTNAAC  
7> GCAACCCAGCTGAACAACAAGATCTGTGATATTGCCATTAACCTGGGCTGGTNGTCTGCAC

1> CATGCAAAGAAGTCCGAGGCATCTGGCTTCTGTTACGTCAATGATATCGTCTTGGCCATC 450  
2> CATGCCTAGAAGTTTGAGGCCTCTGGCTTCTGCTATGTCAACGACATTGTGTTTGGCATC

*Figure 5A (cont.)*

5> NATGCCANGANGTTTNAGGCCTCTGGNTTCTGCTATGTCAACGACATTGTGATTGGCATC  
7> CATGCCAAGAAGTTTGAGGCCTCTGGTTTCTGCTATGTCAACGACATTGTGATTGGCATC

500

1> CTGGAACTGCTAAAGTATCACCAGAGGGGCTGTACATTGACATTGATATTCACCATGGT  
2> CTGGAGCTGCTCAAGTACCACCCTCGGGTGCTCTACATTGACATTGACATCCACCATGGT  
5> CTGGAGCTGCTCAAGTACCACCCTCGGGTGCTCTACATTGACATTGACATCCACCATGGT  
7> CTGGAGCTGCTCAAGTACCACCCTCGGGTGCTCTACATTGACATTGACATCCACCA

550 600

1> GACGGCGTGGAAGAGGcCTTCTACACCACGGACCGGGTCATGACTGTGTCCTTTCATAAG  
2> GACGGGGTTCAAGAAGCTTTCTACCTCACTGACC  
5> GACGGGGTTCAAGAAGCTTTCTACCTCACTGACCGGGTCATGACGGTGTCTTTCCACAA  
10> CTACACCACGGACCGGGTCATGACTGTGTCCTTTCATAAG

650

1> TATGGAGAGTACTTCCCAGGAACTGGGGACCTACGGGATATCGGGGCTGGCAAAGGCAAG  
5> ATACGGGAAATTTACTTNTTCCNGGGGCACAGGTGACATGTTNTGGAAGTTCGGGGGGCA  
10> TATGGAGAGTACTTCCCAGGAACTTGGGACCTACGGGATATCGGGGCTGGCAAAGGCAAG

700

1> TATTATGCTGTAACTACCCGCTCCGAGACGGGATTGATGACGAGTCCTATGAGGCCATT  
3> TACTACTGTCTGAACGTGCCCCTGCGGATGGGCATTGATGACCAGAGTTACAAGCACCTT  
5> GGAGAGTTGGCCC  
10> TATTATGCTGTAACTACCCGCTCCGAGACGGGATTNATGACGAGTCCTATGAGGCCATT

750

1> TTCAAGCCGGTCATGTCCAAAGTAATGGAGATGTTCCAGCCTAGTGCGGTGGTCTTACAG  
3> TTCCAGCCGGTTATCAACCAGGTAGTGGACTTCTACCAACCCACGTGCATTGTGCTCCAG  
8> CCCTATAGTGAGTCGTATTNN  
10> TTCAAGCCGGTCATGTCCAAAGTAATNGAGATGTTCCAGCCTAGTGCG

800

1> TGTGGCTCAGACTCCCTATCTGGGGATCGGTTAGGTTGCTTCAATCTAACTATCAAAGGA  
3> TGTGGAGCTGACTCTCTGGGCTGTGATCGATTGGGCTGCTTTAACCTCAGCATCCGAGGG  
8> TNAAAACATGACTCACTNNGGNTNNNTACGATTGGGCTGCTTTAACCTCAGCATCCGAGGG  
9> AGGT

850 900

1> CACgCCAAGTGTGTGGAATTTGTCAAGAGCTTTAACCTGCCTATGCTGATGCTGGGAGGC  
3> CATGGGGAATGCGTTGAATATGTCAAGAGCTTCAATATCCCTCTACTCGTGCTGGGTGGT  
4> GGA  
8> CATGGGNAATGCGTTGAATATGTCAAGAGCTTCAATATCCCTCTACTCGTGCTGGGTGGT  
9> NATGCTAAATGTGTAGAAGTTGTAAAACTTTTAACTTACCATTACTGATGCTTGGAGGA

*Figure 5A (cont.)*

950  
1> GGTGGTTACACCATTCGTAACGTTGCCCCGGTGCTGGACATATGAGACAGCTGTGGCCCTG  
3> GGTGGTTATACTGTCCGAAATGTTGCCCCGCTGCTGGACATATGAGACATCGCTGCTGGTA  
4> GGTGGCTACACAATCCGTAATGTTGCTCGATGTTGGACATATGAGACTGCAGTTGCCCTT  
8> GGTGGTTATACTGTCCGAAATGTNGCCCCGCTGCTGGACATATGAGACANCGCTGCTGGTA  
9> GGTGGCTACACAATCCGTAATGTTGCTCGATGTTGGACATATGAGACTGCAGTTGCCCTT

1000  
1> GATACGGAGATCCCTAATGAGCTTCCATACaATGACTACTTTGAATACTTTGGACCAGAT  
3> GAAGAGGCCATTAGTGAGGAGCTTCCCTATAGTGAATACTTCGAGTACTTTGCCCCAGAC  
4> GATTGTGAGATTCCCAATGAGTTGCCATATAATGATTACTTTGAGTATTTTGGACCAGAC  
8> GAAGAGGCCATTAGTGAGGAGCTTCCCTAATAGTGAATACTTCGNTACTTTGCCCCAGAC  
9> GATTGTGAGATTCCCAATGGTAAGTGTTCTCATTACAATATCTTTATTGTATG

1050  
1> TTCAAGCTCCACATCAGTCCTTCCAATATGACTAACCAGAACACGAATGAGTACCtGGAG  
3> TTCACACT  
4> TTCAAACCTGCATATTAGTCCTTCAAACATGACAAACCAGAACAC  
8> TTCACACTTCATCCANATGTCAGCACCCGCATCGAGAATCCAGAACTCACGCCAGTATC

1100  
1> AAGATCAAACAGCGACTGTTTGAGAACCTTAGAATGCTGCCGCACGCACCTGGGGTCCAA  
8> NGGACCAAGATCCGCCAGACAATCTTTGNAAACCTGAAGGTTCTTNAACC

1150 1200  
1> ATGCAGGCGATTCTTGAGGACGCCATCCCTGAGGAGAGTGGCGATGAGGACGAAGACGAC

1250  
1> CCTGACAAGCGCATCTCGATCTGCTCCTCTGACAAACGAATTGCCTGTGAGGAAGAGTTC

1300  
1> TCCGATTCTGAAGAGGAGGGAGAGGGGGGCGCAAGAACTCTTCCAACCTTCAAAAAGCC

1350  
1> AAGAGAGTCAAAACAGAGGATGAAAAAGAGAAAGACCCAGAGGAGAAGAAAGAGTCACC

1400  
1> GAAGAGGAGAAAACCAAGGAGGAGAAGCCAGAAGCCAAAGGGGTCAAGGAGGAGGTCAAG

1> TTGGCCTGA

9> F06693  
10> H05234  
11> R21136

**Figure 5B**

```

HD1      ( 1) -----maqTqGTRRKVCYYYDGDVGNYYYGQGHMPKPHRI RMTHNLLLNYGLYRK
RPD3     ( 1) mveyeatpfdpITVKPSDKRRVAYFYDADVGNYYAYGAGHPMKPHRI RMAHSLIMNYGLYKK
x_rpd3   ( 1) -----MALTLGTTKKKVCYYYDGDVGNYYYGQGHMPKPHRI RMTHNLLLNYGLYRK

<EstA>
HD1      ( 51) MEIYRPHKANAEEMTKYHSDDYIKFLRSIRPDNMSEYSKOMQRFNVGEDCPVFDGLFEFC
RPD3     ( 61) MEIYRAKPAKQEMCOFHTDEYIDFLSRVTPDNLEMFKRESVKFNVGDDCPVFDGLYEYC
x_rpd3   ( 51) MEIFRPHKASAEEMTKYHSDDYIKFLRSIRPDNMSEYSKOMQRFNVGEDCPVFDGLFEFC

<EstA>
HD1      ( 111) QLSTGGSVASAVKLNKQQTDI AVNWAGGLHHAKKFEASGFCYVNDIVFGILELLKYHPRV
RPD3     ( 121) SISGGGSMGAARLNRGKCDVAVNYAGGLHHAKKFEASGFCYVNDIVLGI IELLYHPRV
x_rpd3   ( 111) QLSAGGSVASAVKLNKQQTDISVNWSGGLHHAKKFEASGFCYVNDIVLAI LELLKYHQRV

<EstB>
<EstA>
HD1      ( 171) LYIDIDIHHGDGVQEA FYLTDRVMTVSFPQIREIY
RPD3     ( 181) LYIDIDVHHGDGVQEA FYLTDRVMTCSFHKYGEFFPGTGELRDIGVGAGKNYAVNVPLRD
x_rpd3   ( 171) VYIDIDIHHGDGVQEA FYLTDRVMTVSFHKYGEFFPGTGELRDIGAGKGKYYAVNYALRD

<EstC>
<EstB>
HD1      ( 231) GIDDESIEAIFKPVMSKVMEMFQPSAVVLQCGSDSLSGDRLGCFNLIRGHGECVEYVKS
RPD3     ( 241) GIDDATYRSVFEPVIKKIMEWYQPSAVVLQCGSDSLSGDRLGCFNLISMEGHANCVNYVKS
x_rpd3   ( 231) GIDDESIEAIFKPVMSKVMEMFQPSAVVLQCGSDSLSGDRLGCFNLIRGHGECVEFIKT

<9/4>
<3>
HD1      ( 291) FNLPLLMLGGGGYTIRNVARCWTYETAVALDCEIPNELPYNDYFEYFGPDFKLHISPSNM
RPD3     ( 301) FGIPMMVVGGGGYTMRNVARTWCFETGLLNVLVDKDLPYNEYEYYPDYKLSVRPSNM
x_rpd3   ( 291) FNLPLLMLGGGGYTIRNVARCWTYETAVALDSEIPNELPYNDYFEYFGPDFKLHISPSNM

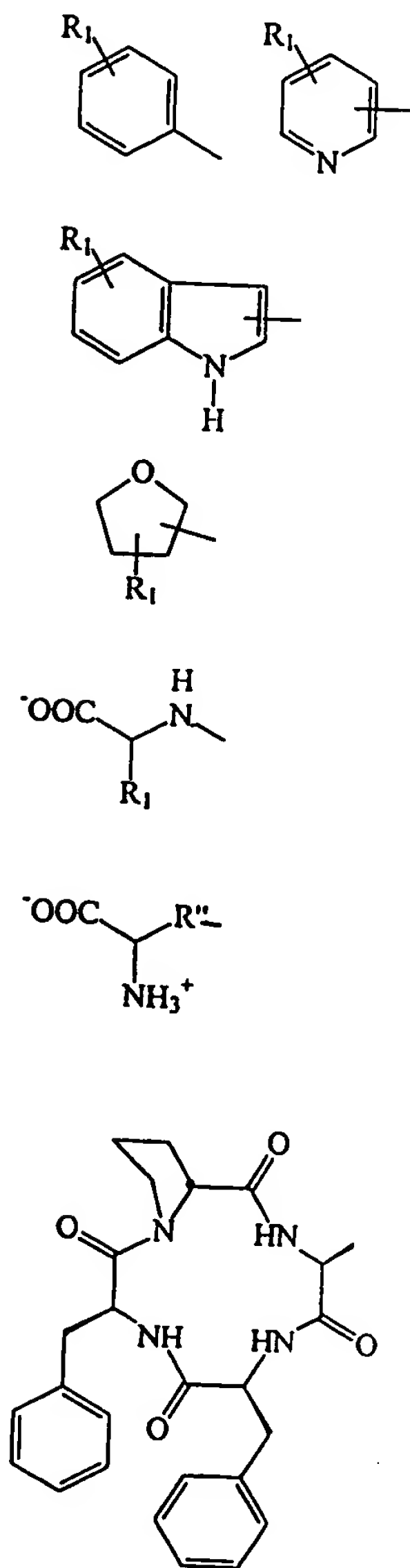
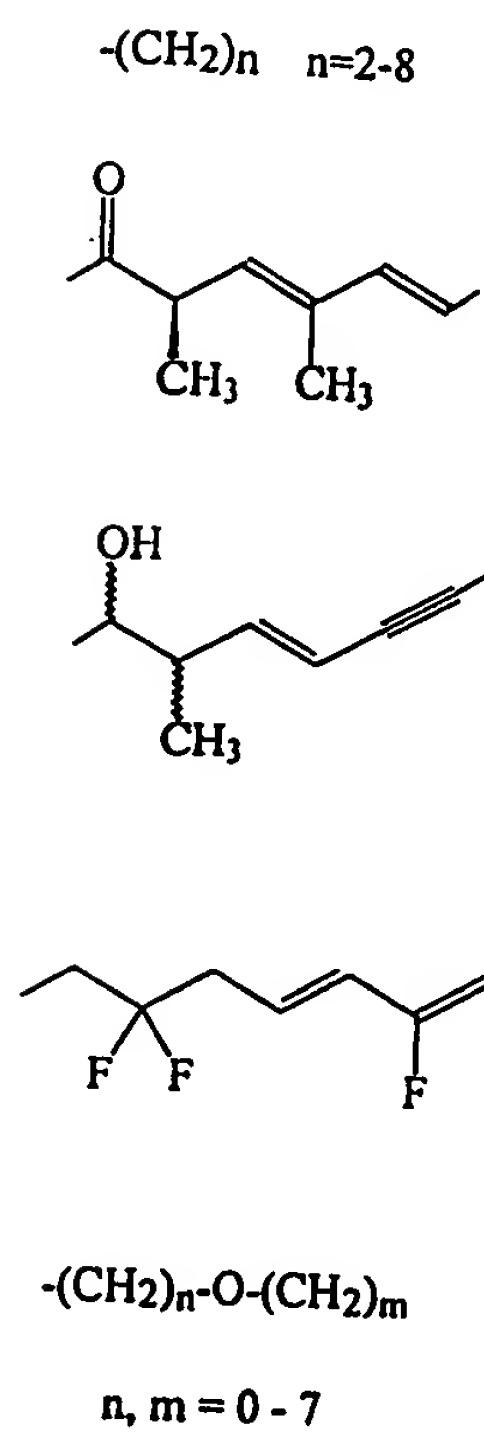
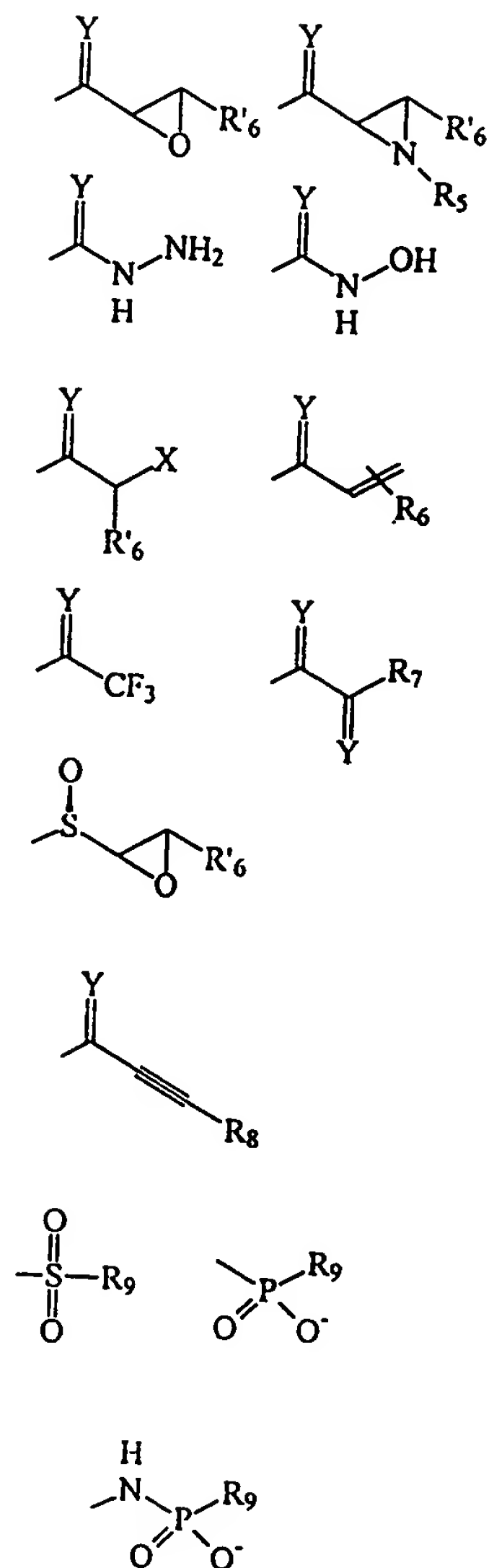
<EstC>
<EstB>
HD1      ( 351) TNQNTNEYLEKIKQRLFENLRMLPHAPGVQMQAIPEDAIPEESGDEDEDDPKRISICSS
RPD3     ( 361) FNVNTPEYLDKVMNTNIFANLENTKYAPSVQLNHTPRDaedlgdveedsaeakdtkggsqy
x_rpd3   ( 351) TNQNTNEYLEKIKQRLFENLRMLPHAPGVQMQAIAEDSIHDDSGEDEDDEDDPKRISIRSS

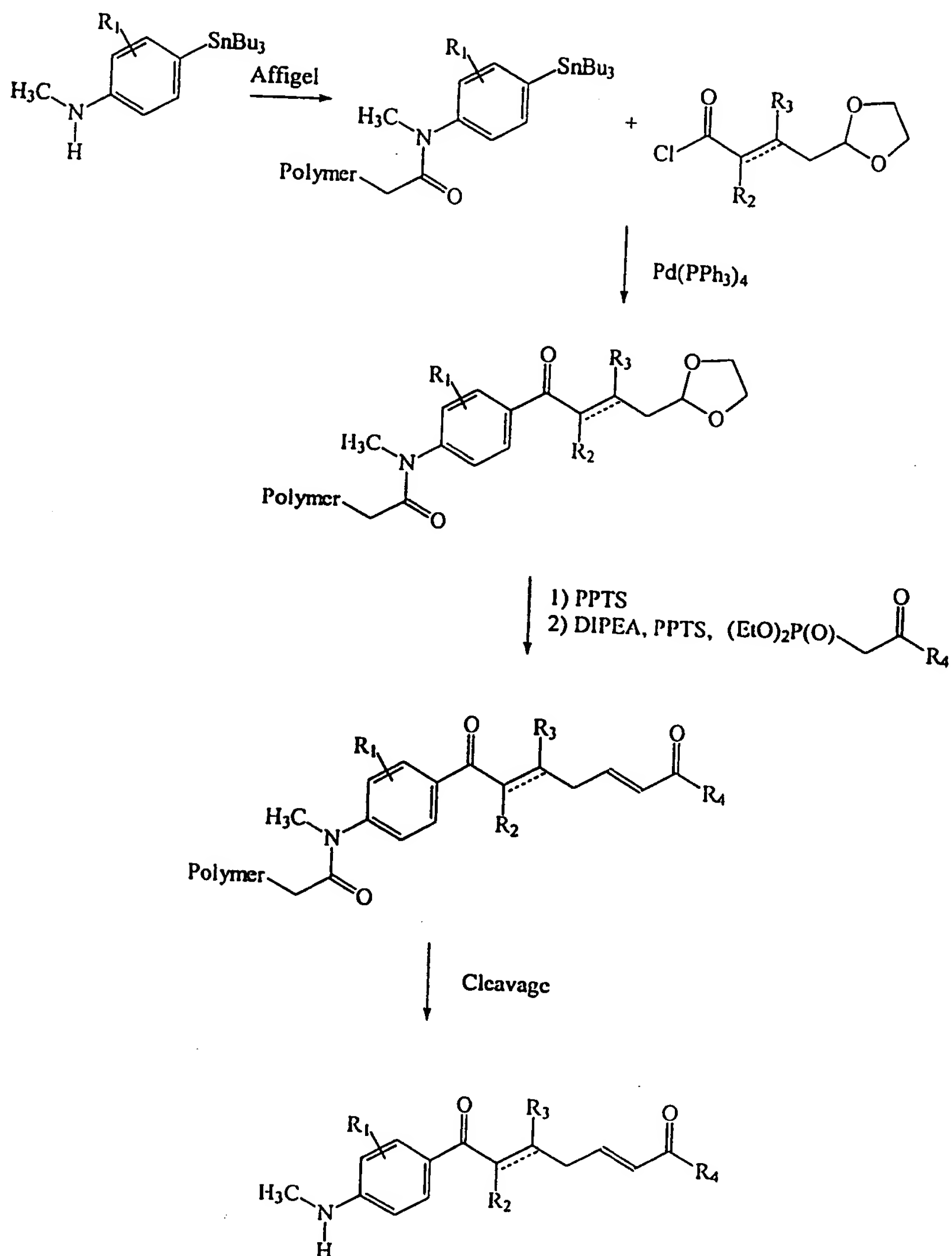
HD1      ( 411) DKRIACEEEFS DSEEEGEGGRKNSSNFKKAKRVKTEDEREKdPEEKKEVTEEEKTKEEK
RPD3     ( 421) ardlhvehdnefy-----
x_rpd3   ( 411) DKRIACDEEFS DSEDEGEGGRKNVANFKKVKRVKTEEEKE--GEDKKDVKEEEKAKDEKT

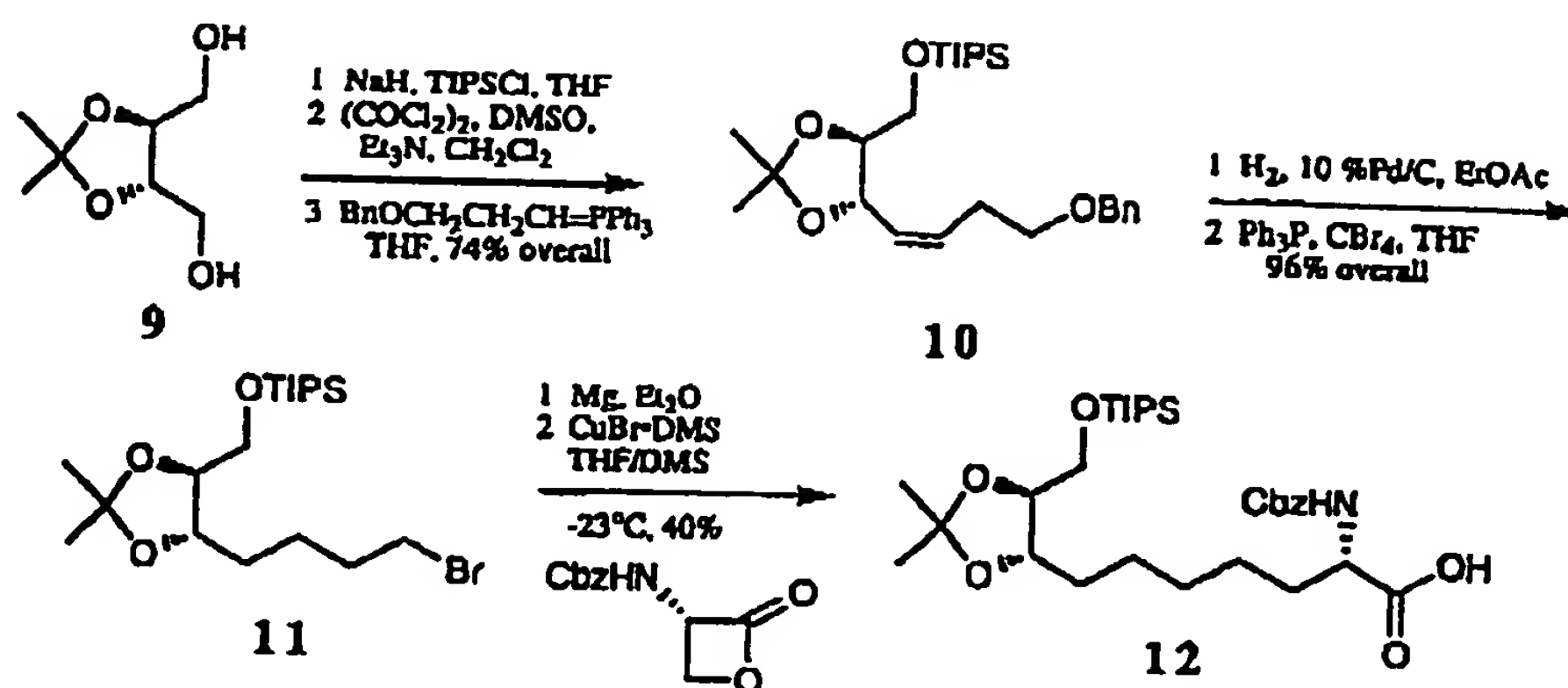
HD1      ( 471) EAKGVKEEVRIa
RPD3     ( 434) -----
x_rpd3   ( 469) DSKRVKEETKsv

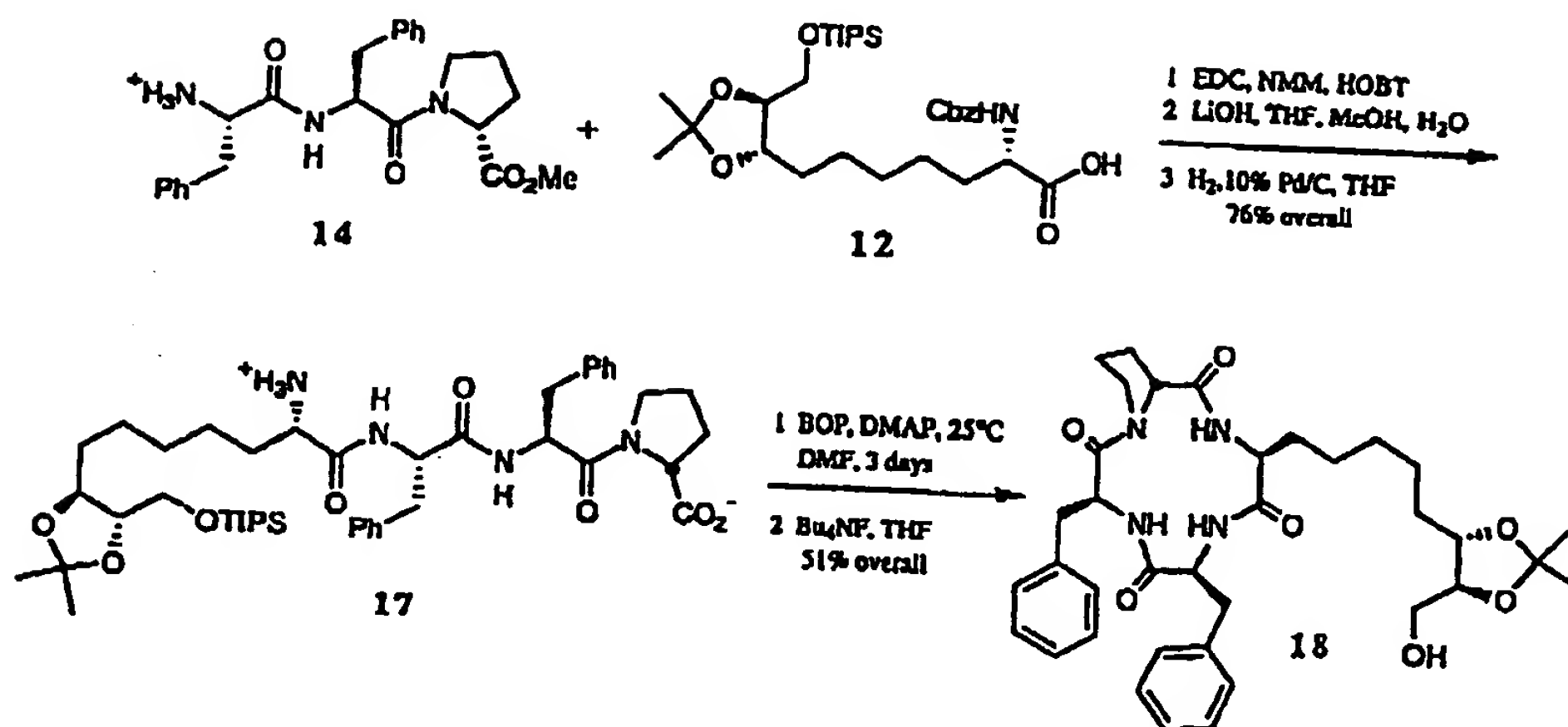
```



**Figure 6****Specificity Element A****Linking Element B****N-Ac Mimic C**

**Figure 7**

*Figure 8A*

*Figure 8B*

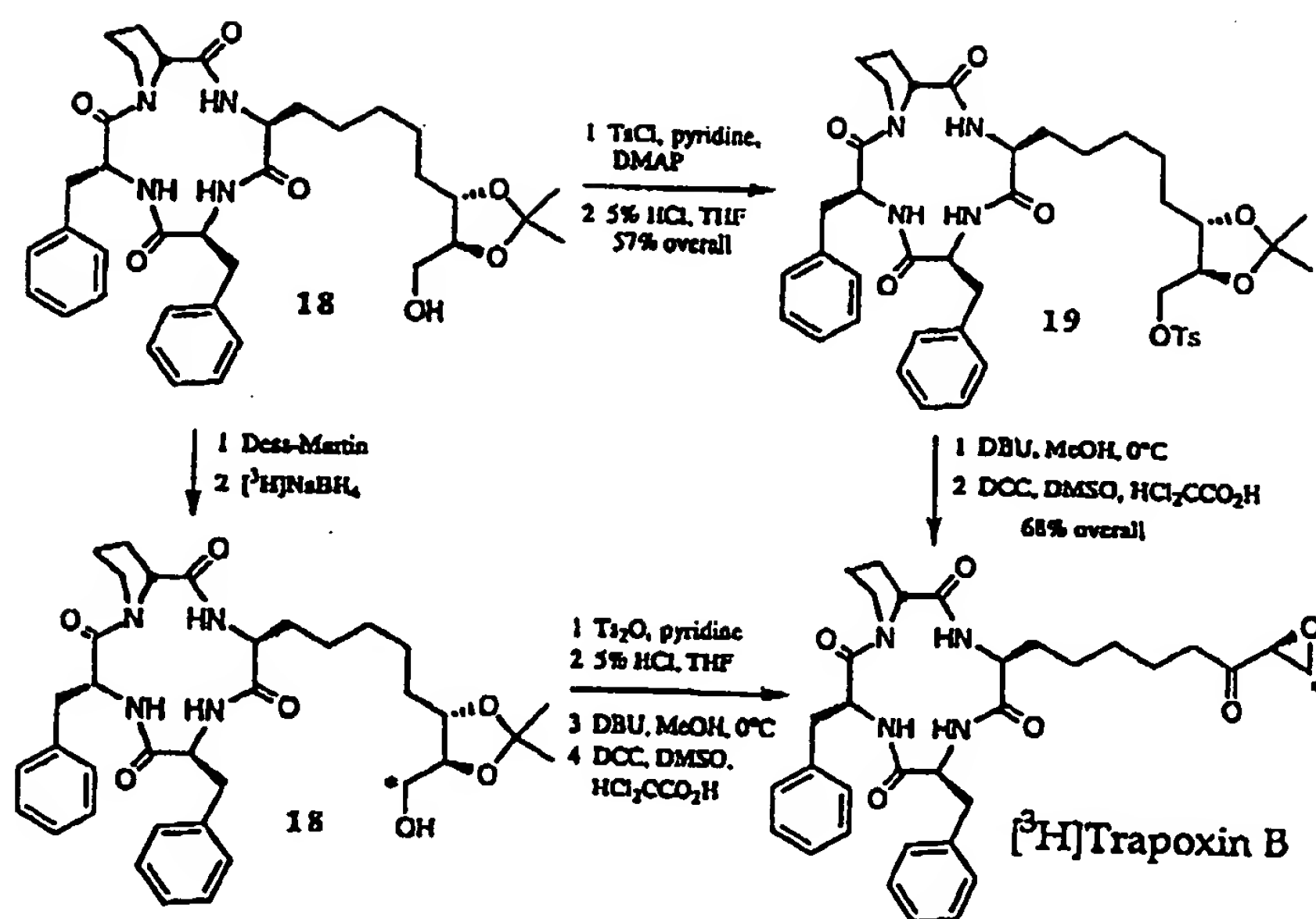
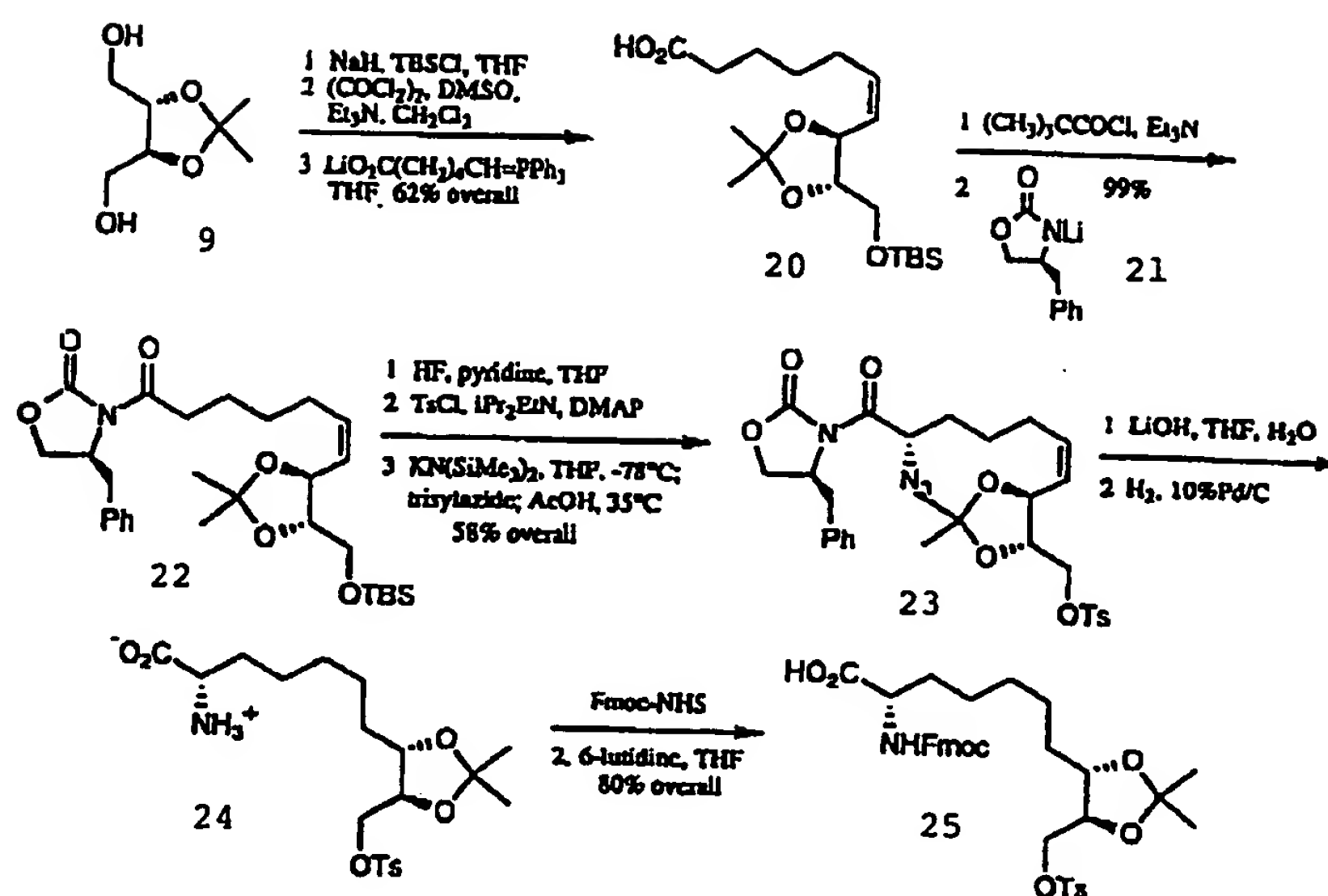
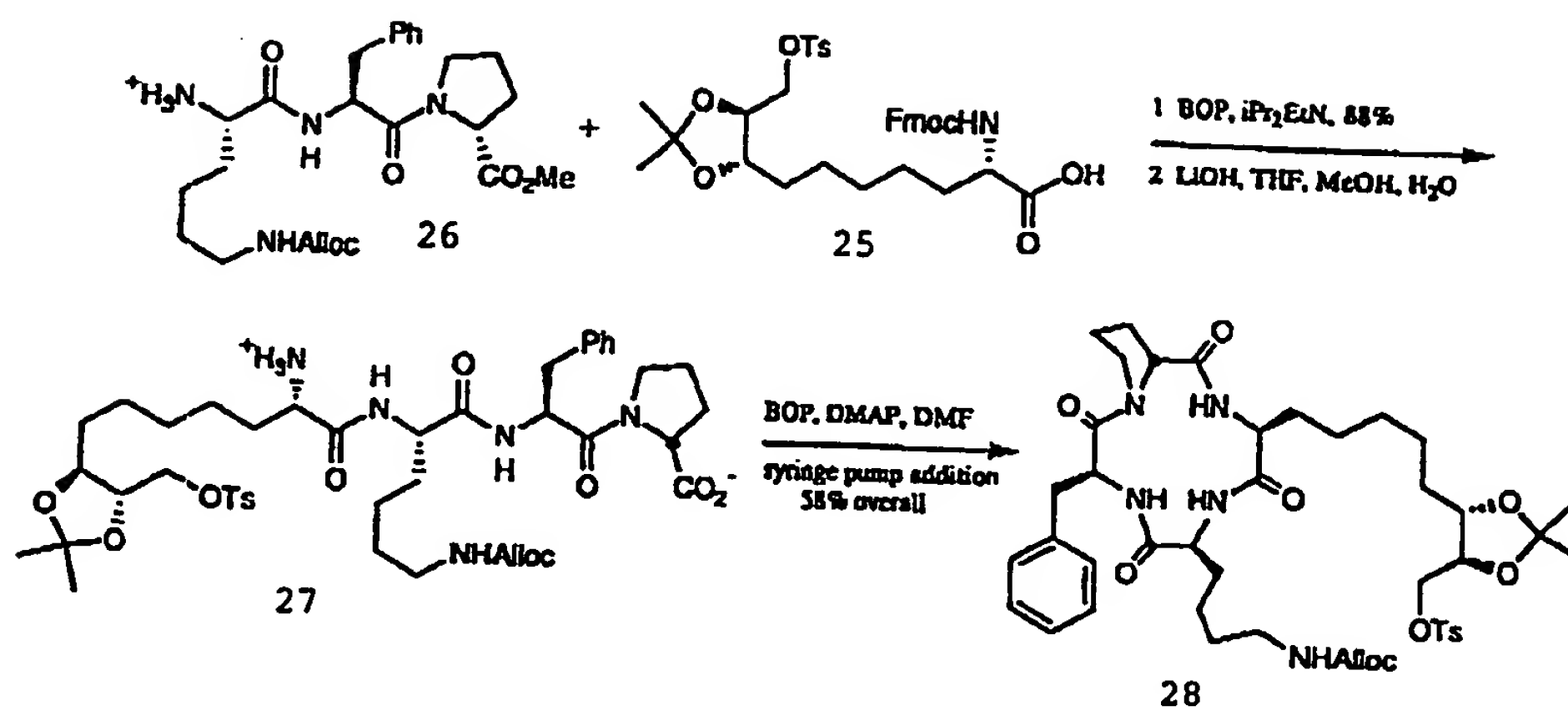
*Figure 8C*



Figure 9A



*Figure 9B*

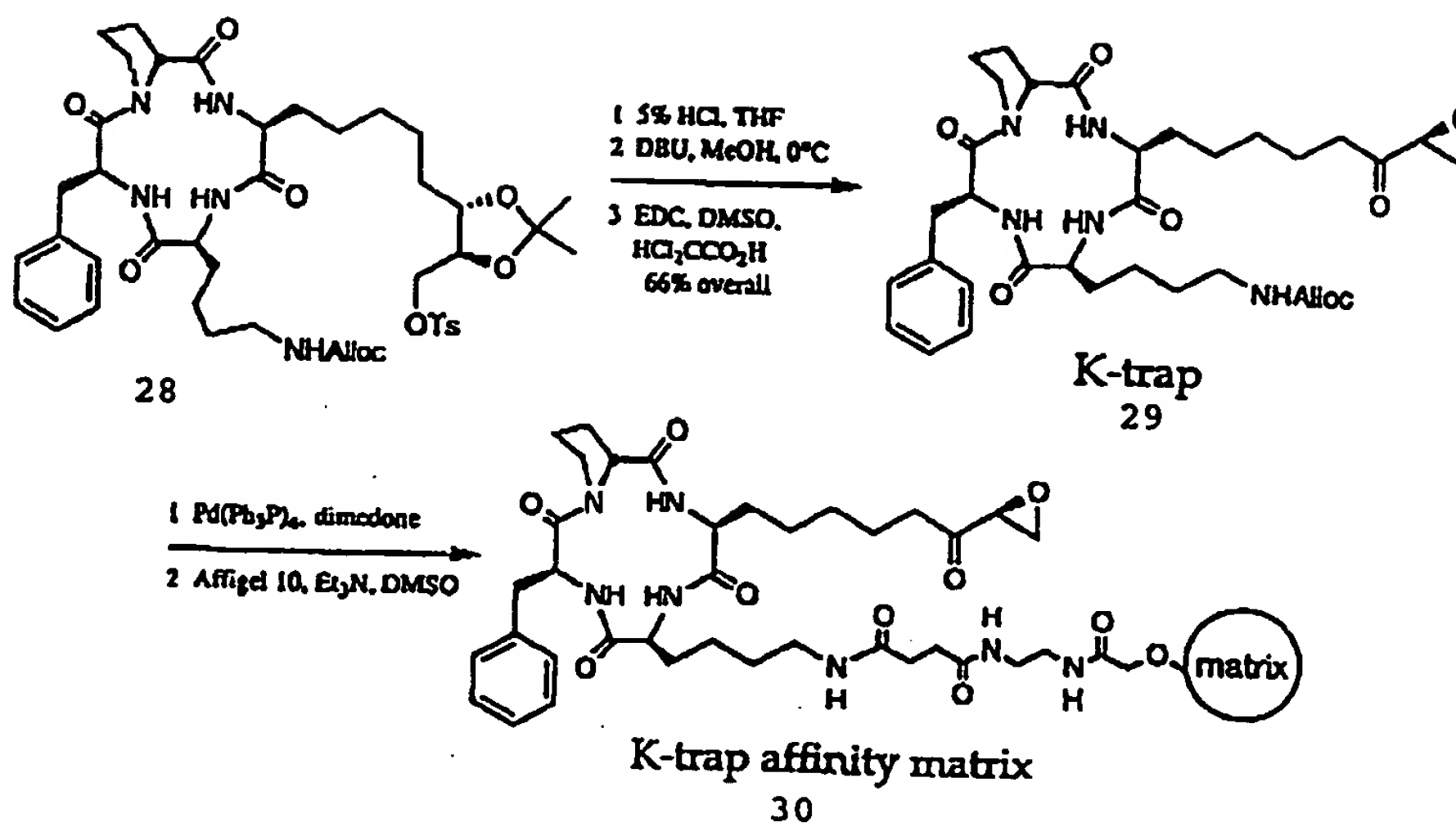
*Figure 9C*

Figure 10A

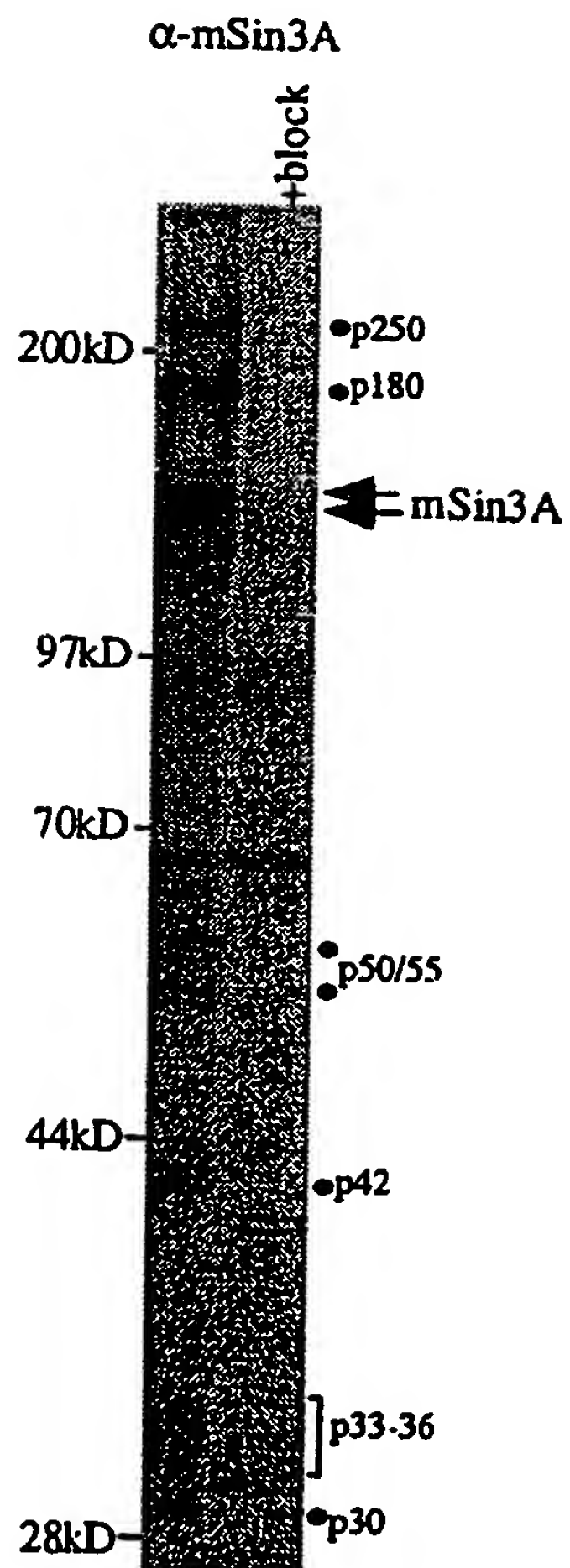


Figure 10B

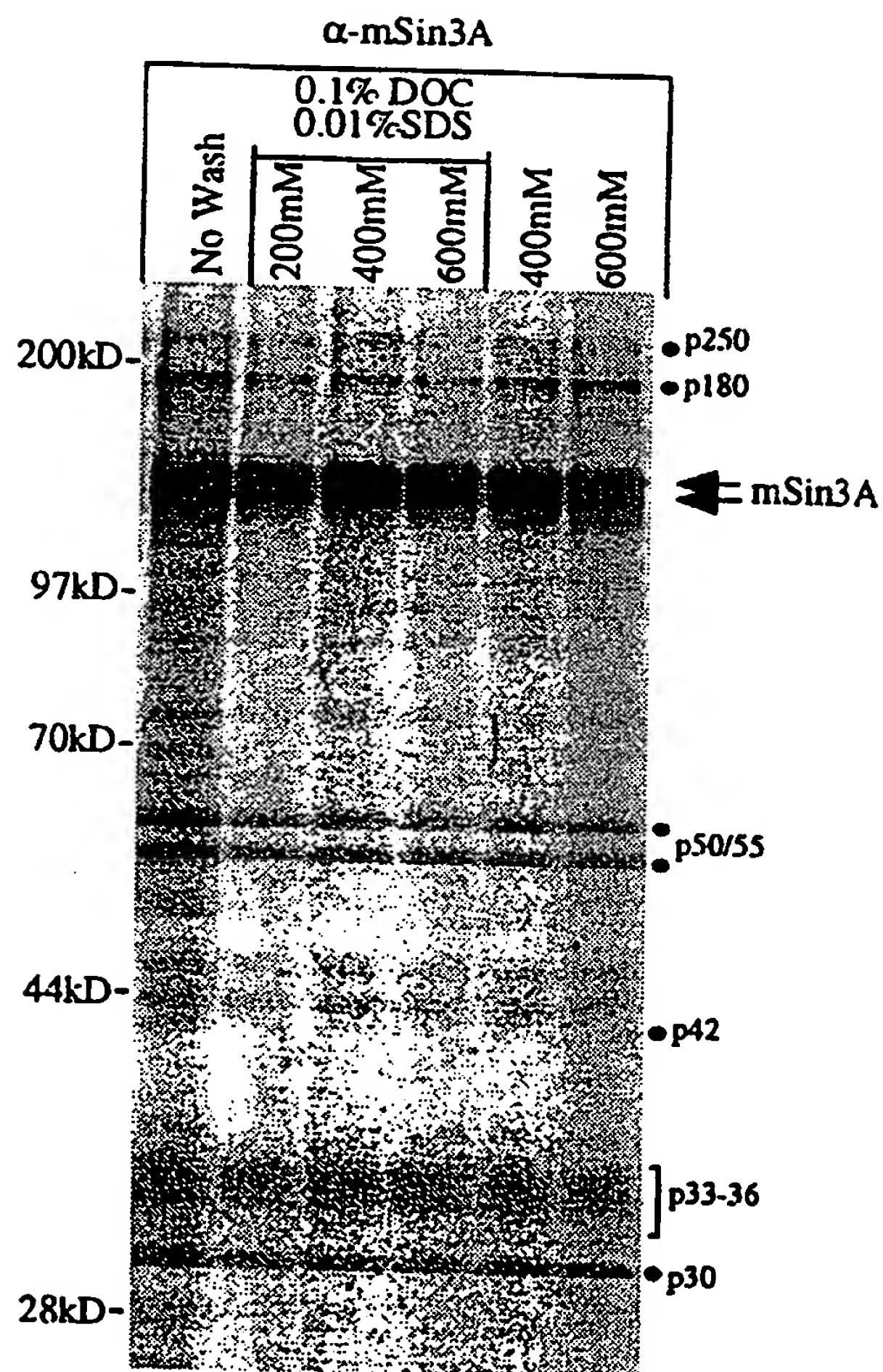


Figure 11A

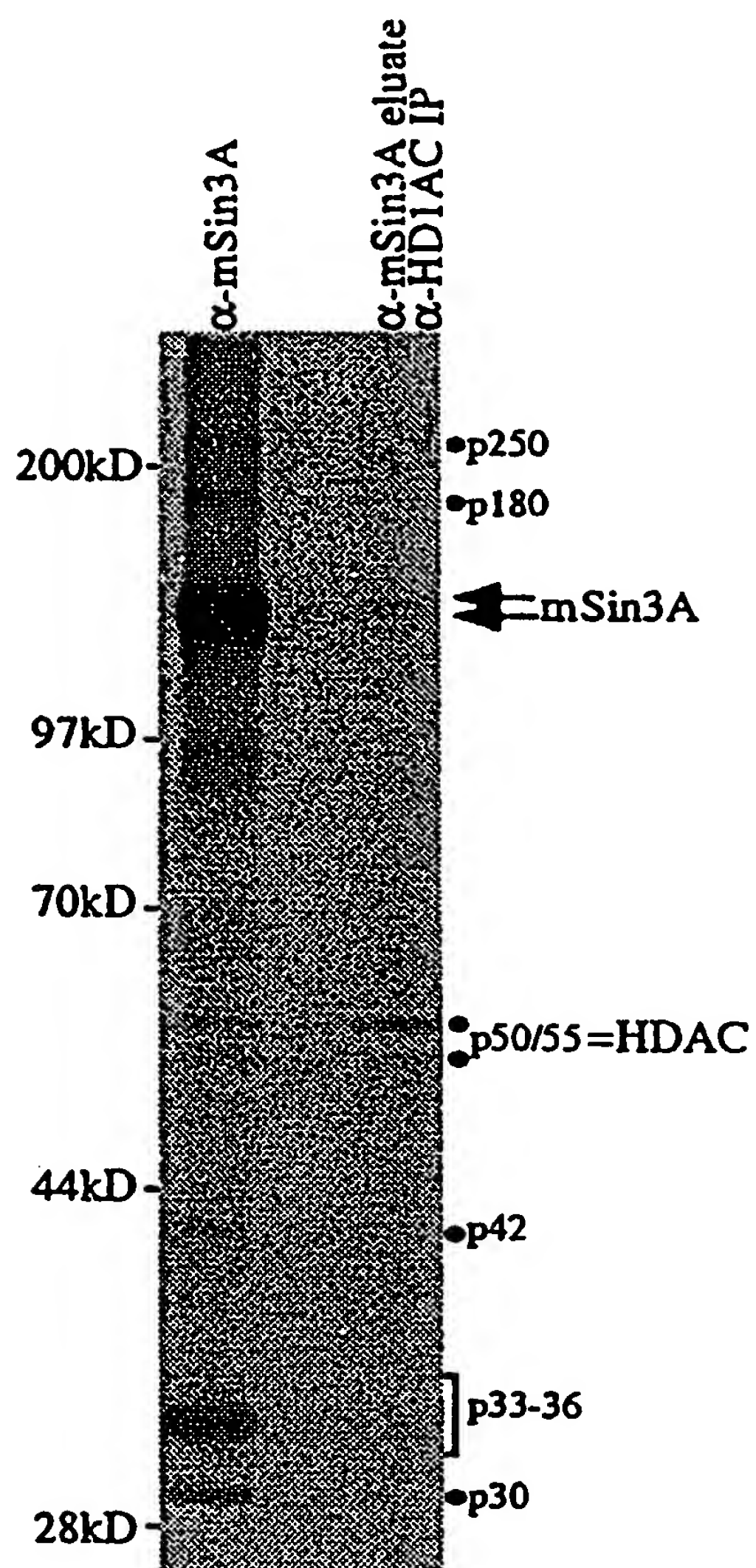


Figure 11B

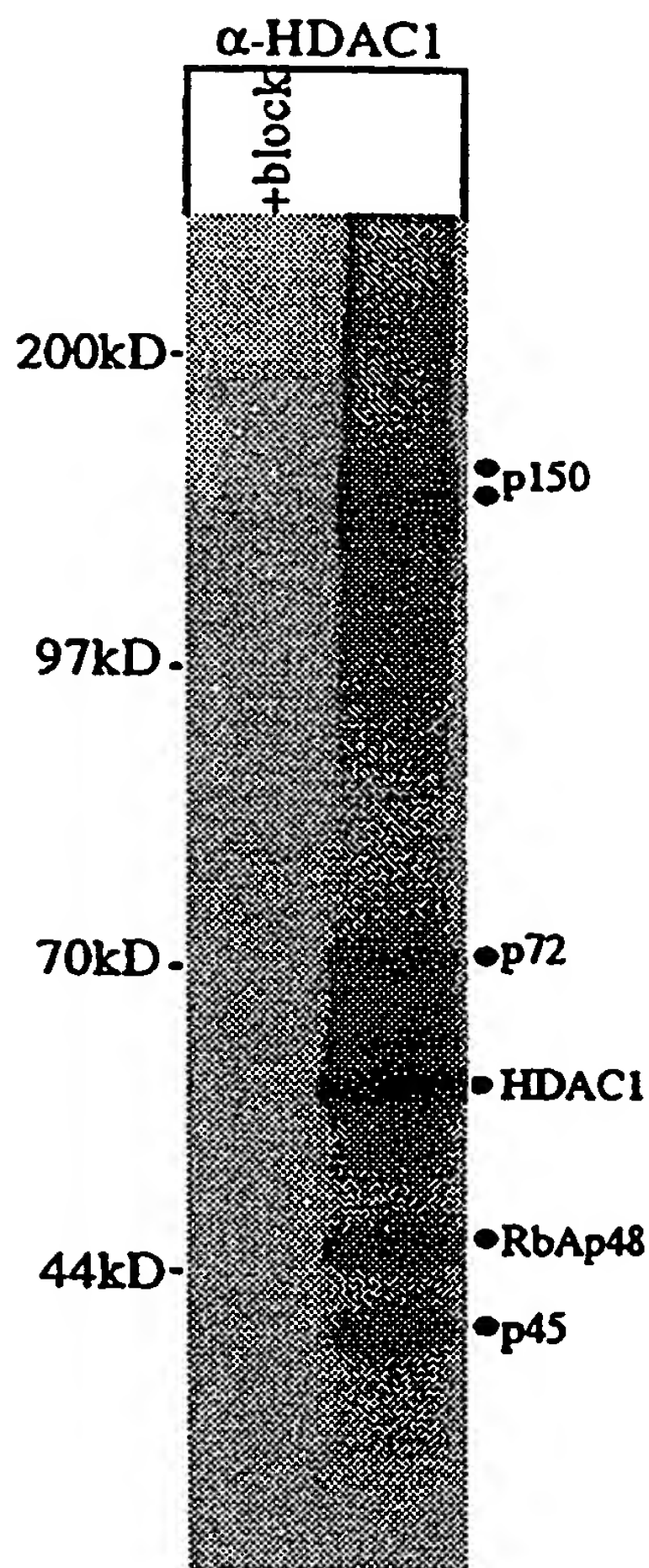




Figure 11C

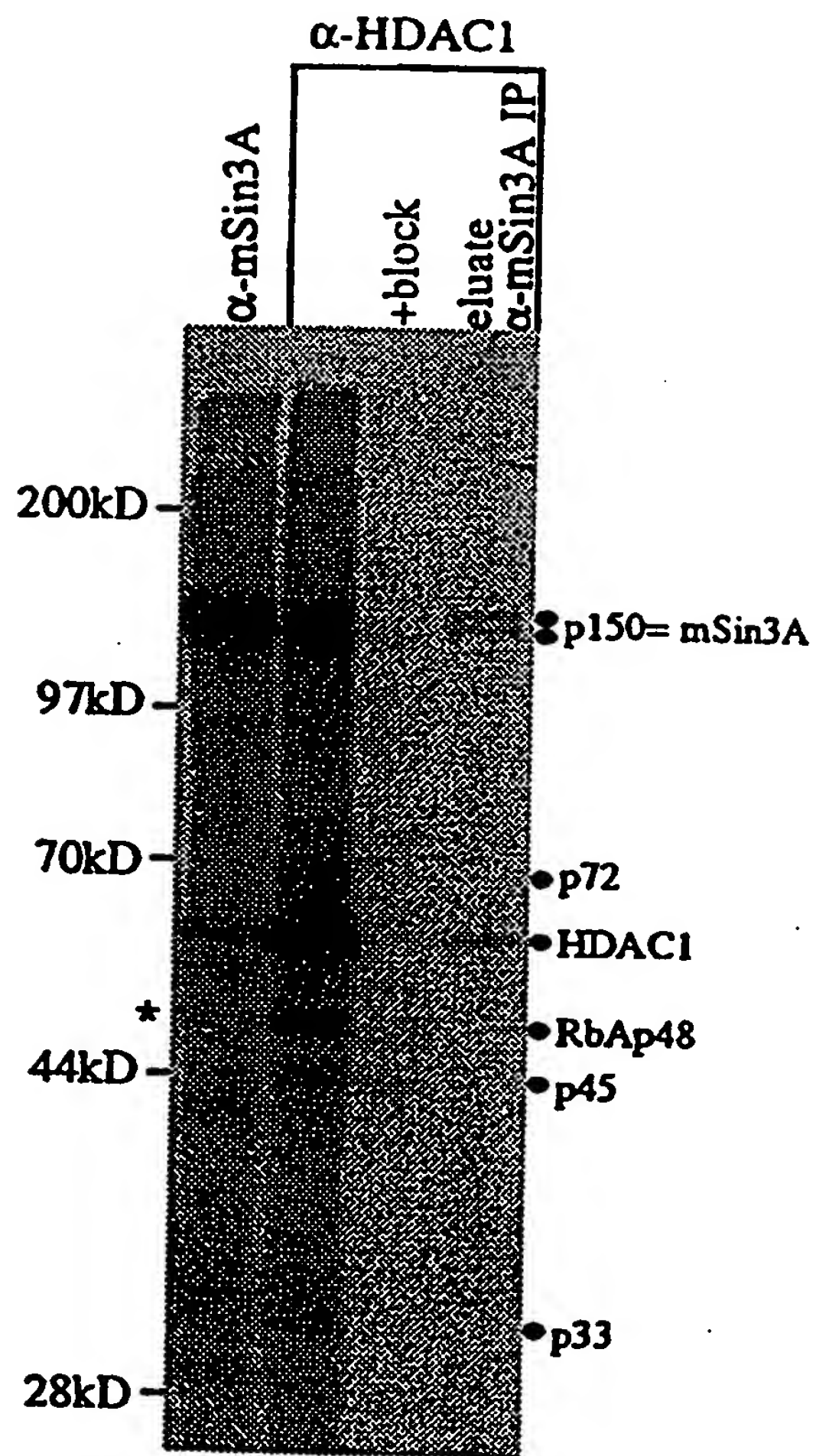


Figure 11D

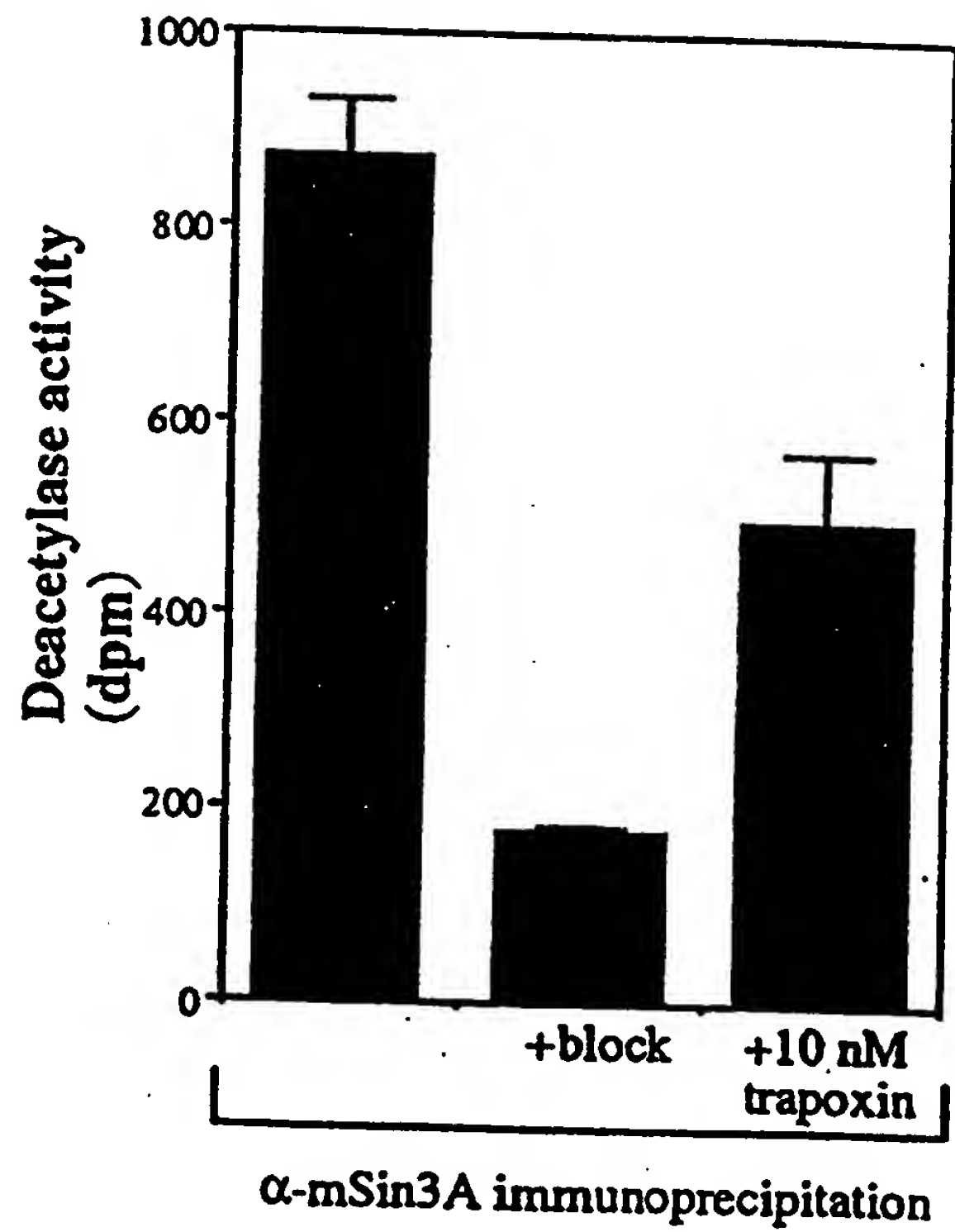


Figure 12A

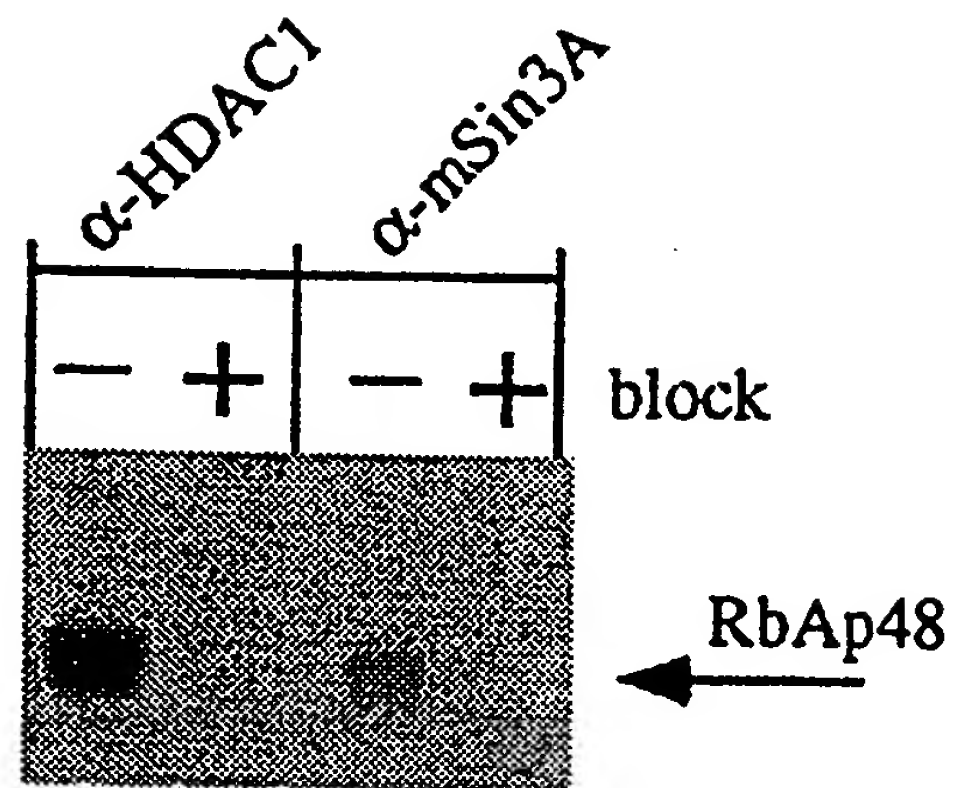


Figure 12B

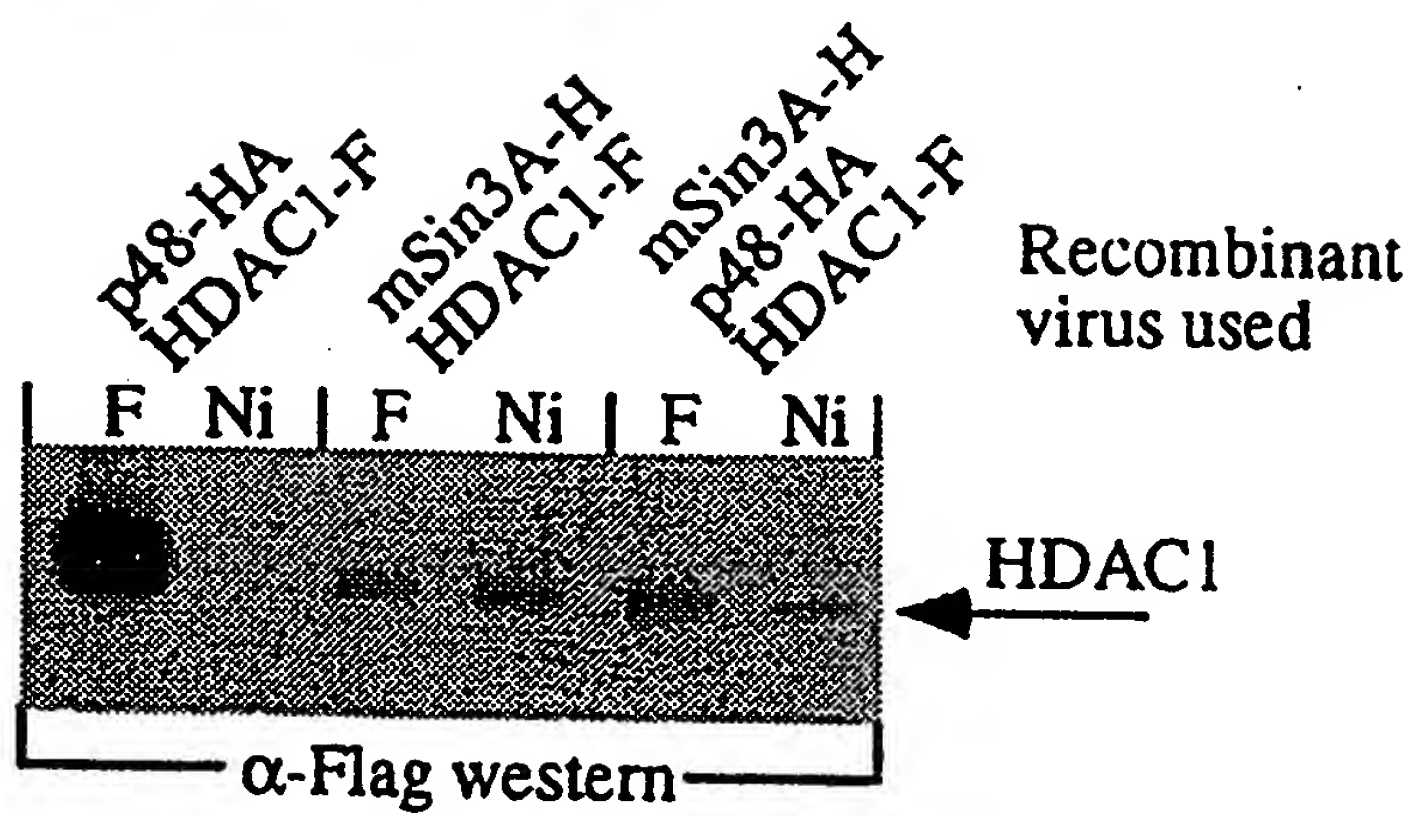


Figure 12C

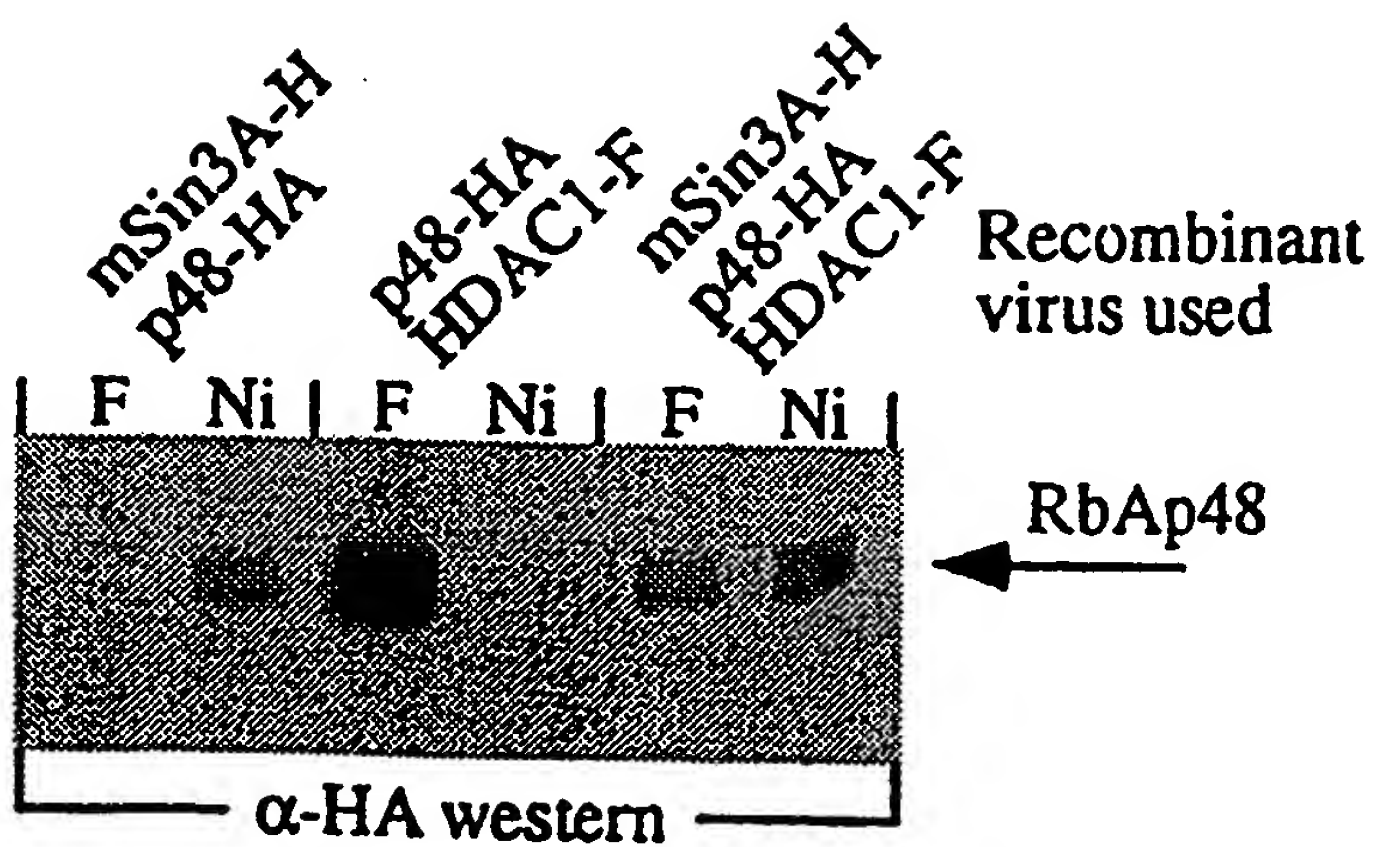


Figure 13A

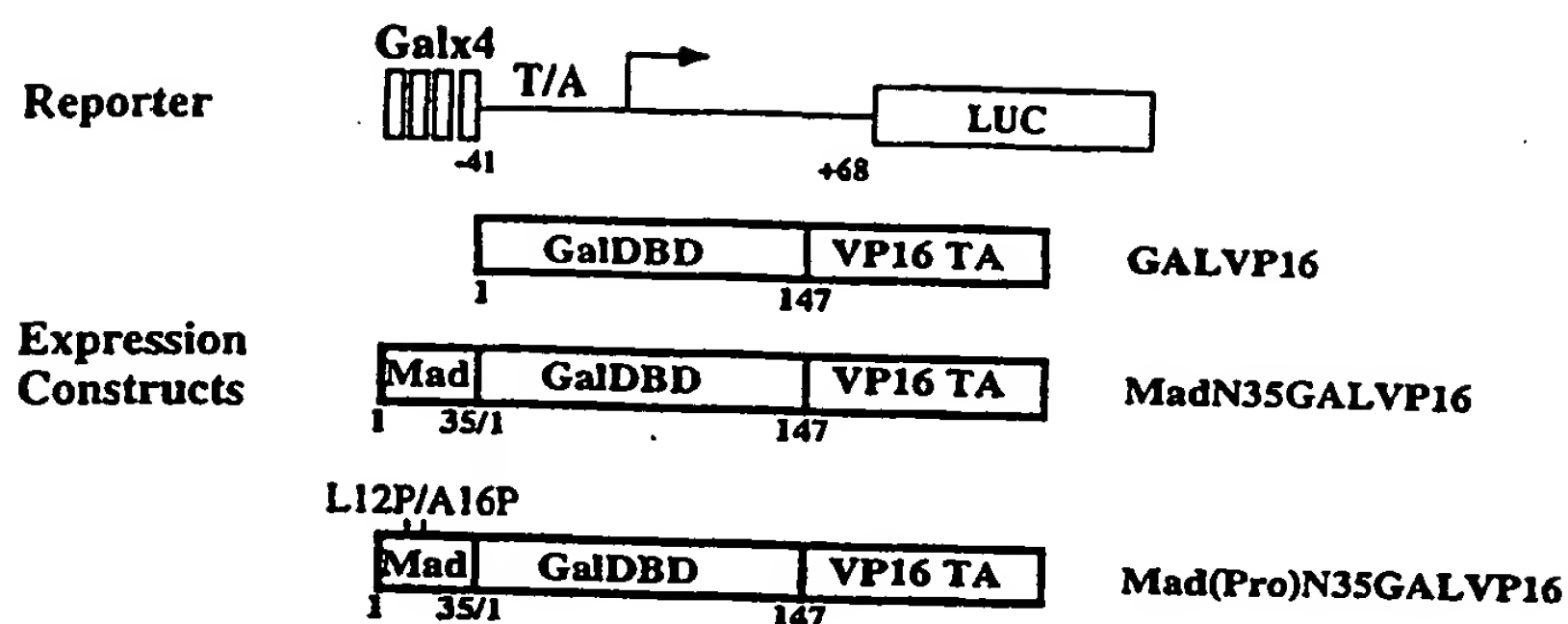


Figure 13B

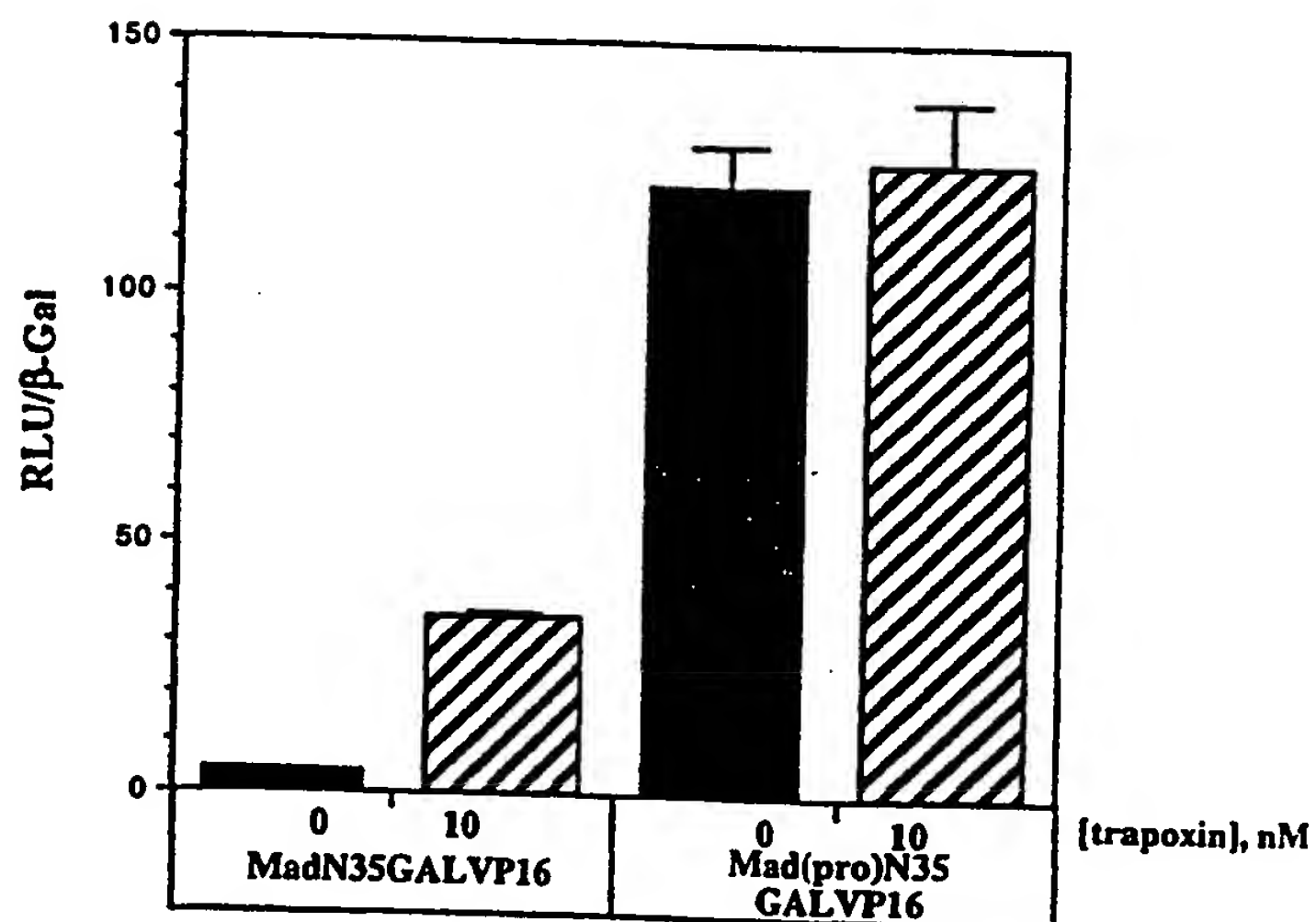
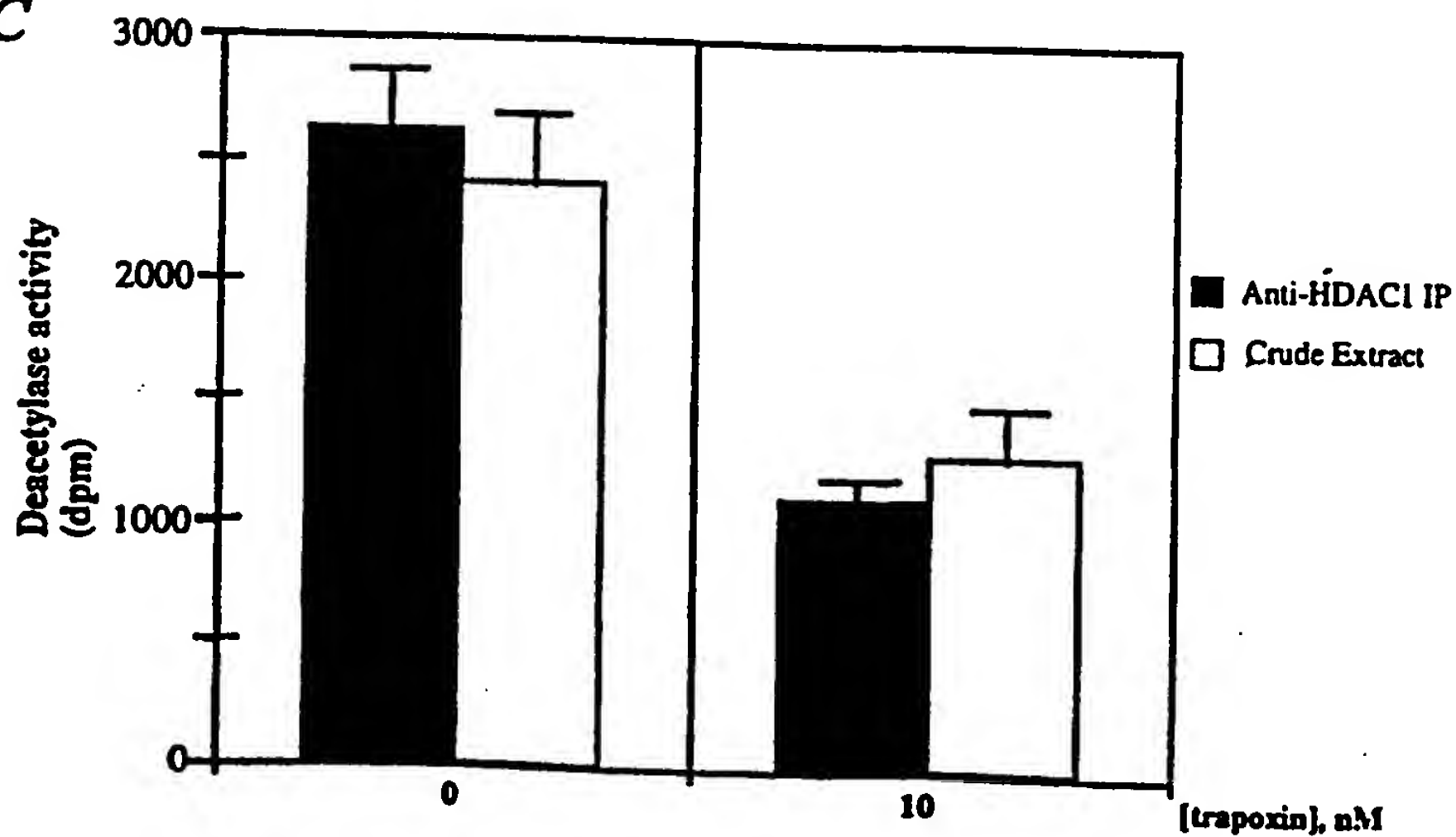


Figure 13C



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/05275

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/55 C12N9/16 C12N15/63 A01K67/027 A61K48/00  
C12Q1/68 A61K38/07 C07K5/12 A61K31/165 C07C229/44  
C07K16/40 C12Q1/44

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01K A61K C12Q C07K C07C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHEMICAL JOURNAL, vol. 303, no. 3, 1 November 1994, pages 723-729, XP002038740 M. MATEO SANCHEZ DEL PINO ET AL.: "Properties of the yeast nuclear histone deacetylase" see abstract see page 725, left-hand column, paragraph 4 - page 726, right-hand column, paragraph 2 see page 728, left-hand column, paragraph 2 - right-hand column, paragraph 2 --- -/-	1,9,50, 51,57, 59-63, 65,66, 68-70

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*Z\* document member of the same patent family

Date of the actual completion of the international search

4 November 1997

Date of mailing of the international search report

16.02.98

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
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Fax: (+31-70) 340-3016

Authorized officer

MONTERO LOPEZ B.

# INTERNATIONAL SEARCH REPORT

Inter. Application No  
PCT/US 97/05275

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 42, 20 October 1995, MD US, pages 24674-24677, XP002038741 SUSANNE KLEFF ET AL.: "Identification of a gene encoding a yeast histone H4 acetyltransferase" cited in the application see the whole document</p> <p style="text-align: center;">---</p>	1-48, 50-73
A	<p>MOLECULAR AND CELLULAR BIOLOGY, vol. 11, no. 12, December 1991, pages 6317-6327, XP002038742 MARC VIDAL ET AL.: "RPD3 encodes a second factor required to achieve maximum positive and negative transcriptional states in Saccharomyces cerevisiae" see page 6317, right-hand column, paragraph 2 see page 6320, left-hand column, paragraph 3 - page 6322, left-hand column, paragraph 2; figure 4 see page 6325, left-hand column, paragraph 4 - right-hand column, paragraph 4</p> <p style="text-align: center;">---</p>	1-48, 50-73
A	<p>JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 265, no. 28, 5 October 1990, MD US, pages 17174-17179, XP000616087 MINORU YOSHIDA ET AL.: "Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by Trichostatin A" see the whole document</p> <p style="text-align: center;">---</p>	57-63
A	<p>JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 30, 25 October 1993, MD US, pages 22429-22435, XP000616088 MASAKO KIJIMA ET AL.: "Trapoxin, an antitumor cyclic tetrapeptide, is an irreversible inhibitor of mammalian histone deacetylase" see the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	57-73



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/05275

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EMBL Database Entry HSU31814 ion number U31814; 19 November 1996 YANG W AL.: "Transcriptional repression b is mediated by interaction with a XP002038746 the yeast global regulator RPD3" cited in the application	25,26
P,X	& PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, no. 23, 12 November 1996, WASHINGTON US, pages 12845-12850, WEN-MIG YANG ET AL.: "Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3" see page 12846, right-hand column, paragraph 1 - page 12847, left-hand column, paragraph 1 see page 12847, right-hand column, paragraph 1 see page 12850, left-hand column, paragraph 2 ---	1-3,7, 11, 25-29, 34-39
P,X	SCIENCE, vol. 272, 19 April 1996, LANCASTER, PA US, pages 408-411, XP002038743 JACK TAUNTON ET AL.: "A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p" see the whole document ---	1-6, 11-24, 26, 28-39,50
P,X	EP 0 708 112 A (CANCER INSTITUTE) 24 April 1996  see page 2, line 53 - page 5, line 20; examples ---	1-7,11, 13-15, 18-20, 22,27, 40, 44-48, 50,51
P,X	CYTOGENETICS AND CELL GENETICS, vol. 73, no. 1-2, 1996, pages 130-133, XP002038744 Y. FURUKAWA ET AL.: "Isolation and mapping of a human gene (RPD3L1) that is homologous to RPD3, a transcription factor in Saccharomyces cerevisiae"  see abstract see page 131, left-hand column, paragraph 3 - page 133, right-hand column, paragraph 2; figure 1 ---	1-7, 12-15, 18-20, 22, 25-27, 40, 44-48, 50,51
	---	-/--

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Inter .nal Application No  
PCT/US 97/05275

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 26, 28 June 1996, MD US, pages 15837-15844, XP002038745 ANDREW A. CARMEN ET AL.: "HDA1 and HDA3 are components of a yeast histone deacetylase (HDA) complex" see abstract see page 15837, right-hand column, paragraph 3 - page 15838, left-hand column, paragraph 1 see page 15839, right-hand column, paragraph 1 - page 15844, left-hand column, paragraph 2</p>	1,12,50
E	<p>--- WO 97 11366 A (MERCK &amp; CO INC ;DULSKI PAULA M (US); GURNETT ANNE M (US); MYERS RO) 27 March 1997</p> <p>see page 3, line 3 - line 10 see page 3, line 19 - page 5, line 8 -----</p>	<p>57,58, 60,61, 63,64, 66,68, 69,72</p>

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 97/05275

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-48, 50-73

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## 1. Claims: 1-48, 50-73

Histone deacetylase HDx, nucleic acid encoding the same, expression vector and host cell containing such nucleic acid, use thereof for producing recombinant histone deacetylase, transgenic animals comprising such nucleic acid or having cells in which a HDx gene is disrupted, recombinant transfection system comprising the HDx nucleic acid, oligonucleotide compositions thereof and test kit comprising the same, antibodies to the HDx polypeptide, diagnostic assays by detection of a mutation of the HDx gene, assays for screening compounds which inhibit histone deacetylation or HDx interaction with cellular proteins.

## 2. Claims: 49, 74-82

Compositions for inhibiting histone deacetylase, pharmaceutical preparations comprising the same and use thereof for modulating growth, differentiation or survival of a mammalian cell responsive to HDx-mediated histone deacetylation.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/05275

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 708112 A	24-04-96	JP 8140687 A	04-06-96
		US 5659016 A	19-08-97
-----	-----	-----	-----
WO 9711366 A	27-03-97	AU 6979096 A	09-04-97
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Form PCT/ISA/210 (patent family annex) (July 1992)

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